Study of immune response to *Mycobacterium tuberculosis* in patients and contacts

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DECLARATION

Statement under O. Ph.D. 8/ (iii) of The Maharaja Sayajirao University of Baroda, Vadodara, India.

The work presented in this thesis has been carried out by me, under the guidance of **Prof. Tamishraha Bagchi**, Department of Microbiology and Biotechnology Centre, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from research studies undertaken by me.

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"Guru Govind Dou Khade, Kaake Lagu Paye,
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Parul Shrivastava

Dedicated to .....

Ma and Papa

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

1. Note: Standard units (SI) of measurements and chemical formulae abbreviations are not included in the list below.

2D	Two-dimensional
Ab	Antibody
Ag	Antigen
ANOVA	Analysis of variance
APC	Antigen presenting cell
APS	Ammonium per sulphate
Bcl 2	B-cell lymphoma-2
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony stimulating factor
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNS	Central nervous system
Con A	Concanavalin A
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte associated antigen
DC	Dendritic cells
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
dNTPs	2'-deoxynucleotide-5'triphosphates
DTT	Dithiothreitol
EDTA	Ethylene-diamine tetra acetic acid
Fas L	tumor necrosis factor receptor superfamily, member 6-ligand
Fas	tumor necrosis factor receptor superfamily, member 6
FITC	Fluorescein isothiocyanate

g	Gram
HHC	Healthy household contacts
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMGT	Immunogenetics
iNOS	Inducible nitric oxide synthase
IPG	Immobilised polyacrylamide gel
kb	Kilo base pair
kDa	Kilo Dalton
KO	Knock Out
LPS	Lipopolysaccharide
M	Molar
MALDI	Matrix assisted laser desorption/ionisation
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
mM	Millimolar
M-MuLV	Moloney murine leukemia virus
MoAb	Monoclonal antibody
mRNA	messenger RNA
Mtb	*Mycobacterium tuberculosis*
PAGE	Polyacrylamide gel electrophoresis
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
pLN	Pulmonary lymph node

plc	Phospholipase C
PPD	Purified protein derivative
PVDF	Polyvinylidine fluoride
qPCR	Quantitative polymerase chain reaction
RBC	Red blood corpuscles
RD	Region of difference
RNA	Ribonucleic acid
RNAsin	RNase inhibitor
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SFU	Spot forming unit
Taq	*Thermus aquaticus*
TB	Tuberculosis
TBE	Tris-Borate EDTA
T_C cells	T cytotoxic cells
TCR	T cell receptor
TE	Tris EDTA
TEMED	NNN'N' Tetramethyl ethylenediamine
Temp	Temperature
TGF	Transforming growth factor
T_H cells	T helper cells
Tm	Melting temperature
TNF	Tumor necrosis factor
TOF	Time of flight
Treg cells	T regulatory cells
Tris	Tri (hydroxymethyl) amino methane hydrochloride
X-gal	5-bromo-4-chloro-3-indoly-β-D-galactopyranoside
αβ T cells	Alpha beta T cells

µg	Microgram
µL	Microlitre

ABSTRACT CUM SUMMARY OF THE THESIS

Thesis title: STUDY OF IMMUNE RESPONSE TO MYCOBACTERIUM TUBERCULOSIS IN PATIENTS AND CONTACTS

Tuberculosis is an ancient disease that still remains a gobal health concern with morbidity of 8.8 million people and mortaility rates reaching 1.4 million in the year 2011. The picture is more gloomy in a developing country like India, which shares the highest global tuberculosis burden along with China. Poverty and thereby malnutrition, overcrowded living conditions, poor vent ilation and poor hygiene habits enhances the risk of transmission of tuberculosis by many folds. The increasing incidence of HIV infection further aggrevates the situation. Tuberculosis is the most common opportunistic infection in HIV-seropositive patients. Recently, the emergence of total drug-resistant tuberculosis is also seen along with extensive-drug resistant and multi-drug resistant tuberculosis. These statistics necessitates the better understanding of immune response to the disease for effective control, diagnosis and treatment.

Alveolar macrophages are the first immune cells to encounter the tubercle bacilli. Following infection the macrophages can themselves kill intracellular bacteria through reactive oxygen and nitrogen intermediates. It can also present the antigens to T lymphocytes. Success of interaction between infected macrophages and T lymphocytes determines the elimination of the bacteria. Many studies have shown the importance of cellular immunity in tuberculosis. Helper T cells can be divided into phenotypes (Th1 & Th2) on the basis of cytokines they secrete. The Th1 phenotype secrete pro-inflammatory cytokine IFNγ and IL-2 whereas Th2 phenotype secret anti-inflammatory cytokine IL-4, IL-5 and IL-10. The Th1 response is indicative of protective immunity while Th2 response represents impaired immunity leading to severe pulmonary tuberculosis. Both $CD4^+$ and $CD8^+$ T cells can produce IFN-γ cytokine. IFN-γ induces autophagy as well as activates macrophages in conjunction with TNF-α to facilitate killing of intracellular mycobacterial through reactive nitrogen and oxygen intermediates. IL-10 which is an anti-inflammatory cytokine produced by macrophages and T cells during Mtb infection, possesses macrophage deactivating properties and decreases IFNγ production. IL-10 is known to antagonize the actions of IFN-γ and dampening the Th1 immune response.

Regulatory T cells (Tregs) are another subset of Th cells, characterized by the expression of transcription factor FoxP3. Tregs are known to dampen the immune response to various pathogens besides *M. tuberculosis*. It is known that Treg cells are expanded in tuberculosis patients as compared to healthy controls and correspondingly Foxp3 expression also helps differentiate different disease states. TGF-β produced by Tregs inhibits the pro-inflammatory responses manifested by the cytokines IL-2, IFN-γ and TNF-α. TGF-β also leads to fibrosis of granuloma which could either be beneficial in containing the infection on the other hand may also lead to increased cavitation and subsequent reactivation.

Following inhalation, the tubercle bacilli may not be killed immediately. In such case, the immune cells are recruited to the site of infection under the influence of cytokines and chemokines. These immune cells aggregate around the infected macrophages. This microaggreate structure is known as granuloma. In an immunocompetent person, an organized granuloma is formed characterized by the formation of IFN- γ secreting Th1 cells. In this case, the granuloma undergoes fibrosis and ultimately calcifies. The tubercle bacilli may remain dormant in such granuloma and such individuals are known as latent tuberculosis infected individuals. However following immunosurvellance the disease may reactivate. In an immunodeficient individual the Th2 response predominates within the granuloma leading to casesous necrosis and hematogenous dissemination of bacilli to other parts of the body. Hence granuloma is a dynamic structure which is a pre-requisite to contain the infection but may also serve as a shelter for the bacteria in case the immune response is compromised. Multinucleate giant cells (MGC) are the hallmark of granuloma. They are formed by the fusion of several macrophages. They have decreased bacterial uptake ability than macrophages but they retain their antigen presenting and oxidative killing property. Thus, they seem to be dedicated in killing of already phagocytosed mycobacteria. Several groups have studied MGC in *in vivo* and *in vitro* models are being studied to gain better understanding immunopathology of disease.

In the light of what is already known in the literature, vaccine development and improved diagnostics for tuberculosis still remains a major challenge. Despite extensive research in the field of tuberculosis immunology several aspects pertaining to immune response and

pathogenesis remain undeciphered. Unraveling the key aspects of the biology of the disease and protective immune response against the disease would help in designing new therapeutic approaches. The rationale of the present study was based on the following observations made from the literature that:

a) Monocytes and granuloma being important factors in disease progression and treatment, it is important to understand their role in tuberculosis. Specifically, it would be interesting to study the differences in expression of various proteins in monocytes of patients and compare them with their respective household contacts and controls.
b) Furthermore, how do the cytokines that are produced in patients, household contacts and controls influence the ability of monocytes in granuloma formation.

Immune response in tuberculosis patients, household contacts and healthy controls

Present study investigated the basal level expression (unstimulated) and in vitro stimulated *M. tuberculosis* specific cytokine response was evaluated. Both Th1 (IFN-γ, TNF-α) and Th2 (IL-10 and TFG-β) type of cytokines and Foxp3 (Forkhead box P3 marker for regulatory T cells) mRNA levels in peripheral blood mononuclear cells (PBMC) was analysed for active TB patients, household contacts (HHC) and controls. Additionally, patient and HHC PBMCs' were stimulated with PHA, Ag85A and CFP-10 peptide. Th1 and Th2 cytokines along with Foxp3 mRNA levels were evaluated.

Increased basal mRNA levels of IFN-γ, TNF-α were observed in patients than controls but not HHC. However IL-10 and TGF-β mRNA levels were increased in patients than HHC. Ag85A and CFP-10 peptide stimulated PBMCs' showed increased mRNA levels of IL-2, TNF-α, IL-10, TGF-β and Foxp3 in patients compared to HHC. IFN-γ ELISPOT assay following stimulation of PBMCs' showed more spots in patient than HHC. A Th1 and Th2 cytokine mRNA level suggests that there is no deficiency in cytokine production by TB patients. Increased expression of Th2 type of cytokine and Tregs (Foxp3) may be dampening Th1 response. The study suggests that FoxP3 could serve as marker for immune status in tuberculosis infection.

***In vitro* multinucleate giant cell formation from monocytes of tuberculosis patients and healthy controls**

Multinucleated giant cells (MGC) are the histologic hallmark of granuloma which is known to limit tuberculosis infection. Both Th1 and Th2 type of cytokines regulate the immune response occurring within the granulomas. The objective of the study was to determine whether tuberculosis patient monocytes differed in their MGC forming ability as compared to healthy controls. In vitro MGC formation was carried out by treatment of monocytes with cytokine containing culture supernatant of ConA or PPD stimu lated peripheral mononuclear cells. IL-2, TNF-α, IL-4, IL-10 and TGF-β cytokine levels were analysed in culture supernatants using ELISA. IL-4 and IL-10 were added to culture supernatant separately and simultaneously along with their respective neutralizing antibodies and their consequent effect on MGC formation was evaluated. MGC formation was significantly low in patient monocytes incubated with autologous culture supernatant as compared to control culture supernatant.

Cytokine analysis of the culture supernatants revealed that while IL-4 levels were similar in patients and controls, increased IL-10 levels were found in patients. Exogenous addition of IL-10 resulted in reduced MGC formation. Contrastingly, when IL-4 was added exogenously, it led to increased MGC formation. The effects of both IL-10 and IL-4 were reversed upon addition of their respective antibodies. The findings suggest that one of the factors contributing to the disease could be the effect of cytokines on the functionality of monocytes, which are crucial in the fight against t he organism. Significantly reduced MGC formation was observed on addition of IL-10. The findings imply an overriding role of IL-10 in MGC formation. The suppressive effect of IL-10 on MGC formation was further confirmed by addition of IL-10 neutralizing antibody. Additionally *in vitro* MGC formation in tuberculosis patients before and after treatment was also analysed. It was also observed that following treatment the cytokine profiles in general tends to move more towards the control profile indicating a reversal of the pathophysiological state of the patients. All the peaks corresponding to the CBA (cytometric bead array) analysis of cytokines in treated patients shift to the position which is concomitant with that observed for controls. This reversal of cytokine secreting

pattern has also been observed by others. Interestingly, it was found that the levels of IL-10 in ConA-SN as well as PPD-SN of patients before treatment were found to be significantly higher than those of patients after treatment and healthy controls. This observation further strengthens the findings obtained in the previous experiment that significantly increased IL-10 seems to be overriding the Th1 response (IL-2 and IFN-γ) and thereby exerting its immunosuppressive role leading to decreased MGC formation.

2d-gel electrophoretic analysis of monocytes from tuberculosis patients, household contacts and controls

Several studies have been carried out to analyse the changes in monocytes following *M. tuberculosis* infection. It has been observed that while monocytes obtained from tuberculosis patients undergo necrosis and apoptosis following infection, the monocytes from healthy controls underwent apoptosis only. It is also known that monocytes from PPD-positive healthy controls underwent apoptosis when exposed to PPD or *M. tuberculosis*, whereas monocytes of TB patients underwent apoptosis as well as necrosis. Apoptosis of monocytes thereby appears to play a role in the protective immune response, whereas necrosis leads to enhanced tissue damage and facilitates bacterial dissemination. It is known observed that the phenomena of necrosis could be reverted in tuberculosis patients following anti-TB treatment.

As proteins play a crucial role in all biological processes of the cells, the study of the protein levels might shed light on various physiological and pathological processes. The term ‘proteome’ refers to all measurable proteins in the cells, whereas ‘proteomics’ refers to the integration of changes in the proteome that reflects the different pathophysiological states. Of late proteomics has been used extensively to identify differences which help in the identification of markers associated with various diseases. Studies have been carried out to determine the differences in proteome during monocyte to macrophage differentiation .

Although monocytes from tuberculosis patients and controls have been studied, no proteomic study of such monocytes appears to have been done. In this study an attempt has been made to detect any differences in the adherent cell population derived from the

peripheral blood mononuclear cells (PBMCs) of patients, House hold contacts (HHC) and healthy controls. The proteomic study was further confirmed by using qPCR and western blot analysis.

Results obtained demonstrate α II-spectrin as a major difference between the proteomes. Alpha II-spectrin has been found to be present in HHC and controls but not in patients. This has been substantiated, with the help of qPCR, taking a housekeeping protein GAPDH as a control.

The results demonstrate that lysates obtained from controls contain the breakdown products of αII-spectrin while they are absent in case of patients. The function of spectrin being maintenance of cell shape and structure, it is surprising to see breakdown products of α II-spectrin in the control samples. This however could be a consequence of *in vitro* differentiation of monocytes to macrophages which has been reported by others. Based on these observations, the reason either intact (in 2D gels) or breakdown products (in both 2D and western blots) of α II-spectrin in patients is not seen is probably because spectrin levels in general are reduced in patients. In the present study, monocyte proteome of tuberculosis patients, HHC and controls revealed that αII-spectrin to be one of the spots downregulated in patients. The decreased expression of αII-spectrin in tuberculosis patients was further confirmed by qPCR and western blot analysis. Therefore, this study suggests the possible role of decreased levels of αII spectrin in the pathology of tuberculosis.

Study of cytotoxic T lymphocytes in patients and controls

The protective role of CD4+ T cells in tuberculosis is already known. Of late there has been increased attention paid to the CD8 subset. Studies conducted in different mice models have demonstrated that CD8 T cells also have a role to play in controlling *M. tuberculosis* infection. The CD8+ T cells recognize the infected cells with the help of peptide presented by MHC class I or HLA (Human leucocyte antigen). Several studies have correlated the HLA alleles with the protection and susceptibility to disease. An in silico study was therefore carried out to identify the antigens with respect to their ability to protect or otherwise. In addition CTL assay with lymphocytes obtained, and nylon wool purified from patients and controls were carried out. The effector cells were derived

from these by in vitro stimulation in the presence of specific peptides and interleukins. These effector cells were then examined for their cytotoxic activity against specific peptide pulsed target cells derived from HLA B-4403 specific B-LCLs. The target cells were calcein stained and cytotoxic activity estimated by the amount of calcein released. Two patients and two controls were identified positive for HLA-B*4403 by gene sequencing and using SSP method. The PBMCs' of these individuals were then used for the generating peptide specific effector cells by incubating them with respective peptides.

When the lysis at this ratio of different peptides was compared it was found that all the peptides except Rv2074 displayed CTL response. In accordance to what is already known in the literature, the peptides of both Ag85B and CFP-10 showed more than 30% specific lysis. It was also observed the % specific lysis of plcC peptide was consistently high in all the cases and showed significantly high % specific lysis in comparsion to other members of phopholipase C family (i.e. plc A and plcB) as well as Ag85B. CFP-10 and plcC showed similar % lysis. It is interesting to note that, while both plcC and plcB genes are expressed at higher levels compared to plcA during the growth of virulent strain, peptide derived from all of them have more or less equal potential in activating the immune system as evidenced by the corresponding target cell lysis ability.

In conclusion the findings of this study indicate whereas the monocytes from patients have the ability to form MGC in vitro, it was seen that cytokines secreted by the lymphocytes contributed to the compromised functionality of the monocytes in forming MGC. Furthermore cytokine analysis demonstrated that IL-10 which is known to subdue MGC formation, had a predominat effect on MGC in the continued presence of IL-4 which is considered to favour MGC formation. Also, the proteomic studies indicated that monocytes from tuberculosis patients have decreased levels of α II- spectrin and therefore might be additionally compromised in their functionality. Analysis of peptide specific CTL response shows that plcC peptide was more effective in inducing a CTL specific immune response in comparison to peptides derived from plcA and plcB.

CHAPTER 1

REVIEW OF LITERATURE

"If you can't explain it simply,
you don't understand it well enough"

Albert Einstein

CHAPTER 1

REVIEW OF LITERATURE

Throughout history, it had always been there, a familiar evil, yet forever changing, formless, unknowable. Where other epidemics might last weeks or months, where even the bubonic plague would be marked forever afterwards by the year it reigned, the epidemics of tuberculosis would last whole centuries and even multiples of centuries. Tuberculosis rose slowly, silently, seeping into homes of millions, like an ageless miasma. And once arrived, it never went away again. Year after year, century after century, it tightened its relentless hold, worsening whenever war or famine reduced the peoples' resistance, infecting virtually everybody, inexplicably sparing some while destroying others, bringing the young down onto their sickbeds, where the flesh slowly fell from their bones and they were consumed in the years long fever, their minds brilliantly alert until, in apocalyptic numbers, they died, like the fallen leaves of a dreadful and premature autumn.

The Forgotten Plague: How the War against Tuberculosis was Won - and Lost

Frank Ryan, 1992

Tuberculosis is a chronic granulomatous disease caused by *Mycobacterium tuberculosis*. It is believed to have occurred even in the era before the beginning of recorded history. Its causative agent, *M. tuberculosis* probably has killed more people than any other microbial pathogen, thereby earning the sobriquet, "Captain Among these Men of Death." The genus *Mycobacterium* originated 150 million years ago (Daniel, 2006). Rene Laënnec (1781–1826) a French physician who invented the stethoscope along with his mentor Gaspard Bayle pioneered in the anatomo-clinical conceptualisation of pulmonary tuberculosis. Laënnec studied the auscultatory findings to diagnose tuberculosis. He also observed that tuberculous matter turned from grey to yellow, which

then liquifed (caseation) and expelled through the airways, leaving a cavity (often calcified) during autopsy (Duffin, 1998). Unfortunately Laënnec himself became a victim and died of tuberculosis in 1826. Subsequently, Robert Koch, in 1882, identified tubercle bacillus as the etiologic agent of tuberculosis. The only method of diagnosing the organism at that time was by stethoscopic examination. The development of tuberculin skin test in 1907 and demonstration of latent tuberculous infection in asymptomatic children was therefore, a major leap in the diagnosis of the disease.

Infection of *M. tuberculosis* in a previously unsensitized, unexposed individual is known as primary tuberculosis. In 95% cases the infection is contained at this stage and the person may become latently infected. A latent tuberculosis infected individual can either remain asymptomatic for a long period of time or the disease can get reactivated following immune surveillance. This type of infection is known as secondary tuberculosis. However, in 5% of the cases the primary infection develops into an active disease. The primary infection usually occurs in the lungs. However it can also localize in pharynx (through tonsils), intestine (terminal ileum) or skin. At this stage it can either be converted into an active disease form or the individual may become latently infected depending upon the immune status.

A major breakthrough in the fight against tuberculosis came in 1908 with the discovery of the BCG (Bacillus Calmette-Guérin) vaccine. Albert Calmette and Camille Guérin subcultured a pathogenic strain of *M. bovis* in a glycerin-bile-potato mixture while studying the effect on its virulence. Eventually after a period of 13 years and having been subcultured more than 230 times, the resulting attenuated strain called Bacille Calmette-Guérin, was found to be avirulent when tested on target animals. Till date BCG remains the only tuberculosis vaccine which is being administered to 100 million children each year. BCG protects children from severe forms of tuberculosis (TB meningitis), for which reason it is still extensively used, although its efficacy varies from 0-77% in protecting adults (Bishai, 2013). Various hypotheses have been forwarded to explain the reasons for the variable efficacy of BCG and include the following:

The masking hypothesis is thought to be one of the reasons for variable efficacy. According to this hypothesis, in case the individual is already exposed to tuberculosis in areas with high background exposure, the natural immunizing effect of background tuberculosis duplicates any benefit of BCG due to a high amount of antigenic similarity between strains of mycobacteria.

Another reason, which is attributed to the genetic variation in BCG strains is the differences between BCG daughter strains. Comparative genomic and transcriptomic analysis by Brosch et al. (2007) revealed extensive variation in gene expression both between early and late BCG daughter strains and with respect to virulent tubercle bacilli. These variations lead to differences in gene expression levels, immunogenicity, and possibly, protection against tuberculosis. The antioxidant production also increased as BCG evolved (Brosch et al., 2007). The findings in these studies revealed that early BCG vaccines may be even more superior to the later ones that are widely used.

Besides this, genetic variation in the population owing to allele distribution like in case of HLA-DR (Brahmajothi et al., 1991), HLA-DQ (Goldfeld et al., 1998) and other gene polymorphisms like Vitamin D receptor (Bellamy et al., 1998) and IFN-γ receptor and natural-resistance-associated macrophage protein 1 (Nramp 1) (Bellamy et al., 1998) have been associated with the susceptibility to tuberculosis in various studies and is postulated to affect the efficacy of BCG.

Exposure of non tuberculous environmental mycobacteria like *M. avium*, *M. intracelluare* and *M. marinum* is also proposed to be one of the reasons for variable efficacy of BCG as it leads to generation of nonspecific immune response to mycobacteria. Administering BCG to someone already exposed to environmental mycobacteria fails to augment the immune response that is already present by preventing the initial multiplication of BCG in host (Brandt et al., 2002). Concurrent parasitic infection is also one of the factors hypothesized to dampen the immune response generated by BCG. This effect may be attributed to a dominant Th2 response during the parasitic infection which might mask the Th1 protective response generated by BCG.

Due to all these reasons affecting the efficacy of BCG and lack of other effective vaccines available, tuberculosis continues be one of the major health problems of the world. The World Health Organization (WHO) has estimated 8.7 million new cases and 1.4 million deaths from tuberculosis annually. At least, 1.1 million of these TB cases are HIV (Human immunodeficiency virus) infected. To make the matters worse, 3.7% of the new TB cases are MDR-TB (multidrug-resistant TB) and of these MDR-TB cases, 9% are XDR-TB (extensively drug-resistant TB) cases. MDR-TB is defined as tuberculosis that is resistant to the first-line treatment anti-TB drug groups i.e. isoniazid (INH) and rifampicin (RMP) whereas XDR-TB refers to MDR-TB resistant to the major second-line drugs groups i.e. fluoroquinolones and injectable drugs (WHO factsheet, 2012). India alone accounts for 25% of the world's TB cases and almost 60% of MDR-TB cases are from India, China and the Russian Federation. These statistics and the failure of BCG to confer complete protection against TB necessitate the need for true correlates of tuberculosis immunity (WHO factsheet, 2012).

1.1 PATHOGENESIS OF TUBERCULOSIS

The standard test used for determining whether a person is infected with tuberculosis is the Mantoux tuberculin skin test (TST). It is a test that determines whether a person is infected with *M. tuberculosis*. Tuberculin purified protein derivative (tuberculin PPD) is obtained by precipitation of the heated products of the culture, consisting of *M. bovis* and /or *M. tuberculosis* lysate and is capable of generating delayed type of hypersensitivity response. Standard dose of 5 tuberculin units (TU) is injected intradermally in the forearm and read 48-72 hours later. The reaction is interpreted by measuring the diameter of induration caused due to delayed type of hypersensitivity reaction.

An induration of 5 mm is considered positive in individuals infected with HIV, recent contact with TB patient, persons having fibrotic changes in chest X-ray with old healed TB, or patients who have undergone organ transplants and other immunosuppressed patients. An induration of more than or equal to 10 mm is considered positive in case of children less than four years of age or if the individual is a recent immigrant from high

prevalence country, injection drug user, healthcare worker, Mycobacteriology laboratory personnel, person with high risk like diabetes, prolonged corticosteroid therapy, leukemia etc. that place them at high risk. An induration of 15 mm or more is considered positive in individuals with no known risk factors for TB (CDC factsheet 2011). The asymptomatic latent tuberculosis infected subjects can also be diagnosed by a positive TST.

The course of infection of tuberculosis initiates from the inhalation of respiratory droplet nuclei (1 to 2 mm or less in size) facilitating the entry of tubercle bacilli into the body via the respiratory tract. Larger droplets are however expectorated form the lower respiratory tract due to the physical barriers of nasopharynx and upper respiratory tract. Once the organism manages to reach the lungs they have four potential fates. In an immunocompetent person, the host can spontaneously kill the tubercle bacilli effectively and such individuals have no chance of developing the infection in future. In some cases however, the organisms begin to grow and multiply leading to primary tuberculosis. Containment of infection at this stage is known as latent infection. Latent tuberculosis infected individual may remain asymptomatic throughout life with the bacteria remaining in a dormant state or when the immune response is compromised it can cause reactivation tuberculosis. Such individuals give positive tuberculin skin test. However, infection in an immunocompromised individual can directly develop in to active disease (Schluger and Rom, 1997; Kaufmann, 2001) which may progress along several different pathways as follows:

Progressive pulmonary tuberculosis: In this case the enlargement of the apical region of lungs occurs with the expansion of the area of caseation. Erosion into the bronchus evacuating the caseous center leads to formation of ragged irregular cavity lined by caseous material. Hemoptysis may also occur following the evasion of blood vessels. The infection may spread to the uninfected tissue by direct expansion of the lesion, through

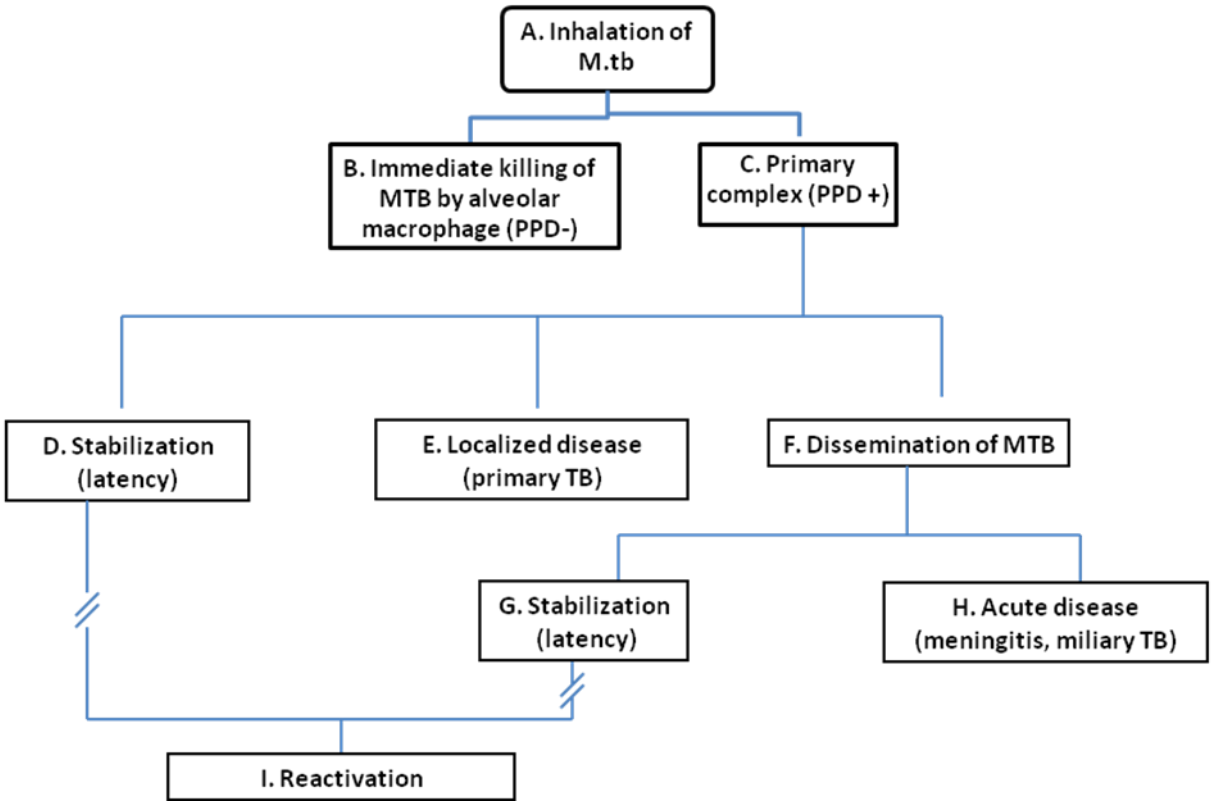


Figure 1.1: Etiology of tuberculosis. (A) Following inhalation of droplet nuclei containing *M. tuberculosis*, the bacilli may be spontaneously killed by the alveolar macrophages (B) or it may survive thus forming a 'primary complex' (C) constituting small infiltrate and draining into lymph nodes. Calcified lesions may be observed on radiographic examination and the PPD-skin test which is a marker for *M. tuberdculosis*-specific T-cell response, becomes positive. In most of the cases the infection stabilizes at this stage and the bacilli may remain dormant (D). However, in few cases it may develop into active disease which is either localized (E) or disseminated to various organs/tissue of the body (F). The disseminated infection may also either get stabilized (G) or develop into active form of disease (H). Failing immune surveillance the dormant bacilli may reactivate later in life (I).

airways and lymphatics or hematogenous spread may lead to miliary pulmonary tuberculosis.

Endobronchial, endotracheal and laryngeal tuberculosis: In this type, the infective material spreads either through lymphatic channels or from expectorated infectious material. Several minute granulomatous lesions are formed along the mucosal lining, which are sometimes only apparent on microscopic examination.

Systemic miliary tuberculosis: Dissemination of infection in this case occurs when the pulmonary venous return to the heart is seeded by infective foci, which leads through the systemic arterial system. The systemic miliary spread can occur in almost any part of the body e.g. liver, bone marrow, spleen, adrenals, meninges, kidney, fallopian tube and epididymis.

Isolated-organ tuberculosis: Unlike systemic miliary tuberculosis in this type of infection, miliary dissemination takes place and appears in any one of the organs/tissue. The organs that are typically involved in this type of infection are meninges (tuberculous meningitis), kidney (renal tuberculosis), adrenals (important cause of Addison's disease), bones (osteomyelitis) , fallopian tubes (salphingitis) and vertebrate (Pott's disease).

Intestinal tuberculosis: In the past drinking of contaminated milk was one of the most common reasons of infection with *M. tuberculosis*. It involves oropharyngeal lymphoid tissue along with spread to the lymph nodes in the neck. Today, most often intestinal tuberculosis is either a complication of advanced tuberculosis or consequent to swallowing coughed-up infective material. The organisms are typically trapped in the mucosa of the small and large intestine, particularly in the ileum.

1.2 IMMUNE RESPONSE TO TUBERCULOSIS

The vertebrate immune system protects against any infection by means of employing either the innate immune system or the adaptive immune system, depending on the situation that the host finds itself in. Furthermore, in case of an adaptive immune

response, the host has the additional choice of utilizing either the humoral or cell mediated arm of the adaptive immune response. We know that within the cell mediated arm of the adaptive immune response there are different paths that the immune system can resort to, such as either that involving the T helper system or the cytotoxic T cell system. Both these are mediated through soluble mediators such as cytokine and chemokines, and direct contact in the case of CTLs.

1.2.1 Innate immunity

Innate immunity is the first defense of the body against any infection. Many studies based on rabbit, mice and human models have emphasized on the role of innate immunity in tuberculosis. In studies on mice, it was observed that the mycobacterium susceptible mice had 20-30 folds more viable mycobacteria than the mycobacterium-resistant mice, seven days after the primary infection through inhalation of *M. tuberculosis*. This difference in the immune response was attributed to the innate immunity, as the T-cell immunity develops only two to three weeks after infection (Dannenberg, 1994). Recently, Subbian et al. (2013) attempted to understand the difference in outcome when virulent *M. tuberculosis* strain and vaccine strain were used in a rabbit model. They observed that the outcome of infection and the progression of the disease depended on the initial events taking place in the infected lung. In study on mice, It was seen that the outcome is influenced by the differential regulation of inflammation associated innate immune cells and related gene expression pattern. For e.g. in case of virulent strains, along with the increase in gene expression for inflammatory markers like STAT1, there was also recruitment and activation of macrophages, PMN, and fMLP (N-formyl-Methionyl-Leucyl-Phenylalanine)-stimulation.

Human studies also reiterate the importance of innate immunity in tuberculosis infection. Naturally acquired T-cell immunity fails to counter the exogenous reinfection of the lung (Henderson et al., 1997). Recent study on thymocytes of neonates reported a novel population of innate T cells (αβTCR+ Thymocytes) that would provide early source of IFN-γ and facilitate generation of adaptive immune response (Gold et al., 2008).

Studies have demonstrated that endocytosis of *M. tuberculosis* can either occur through nonopsonized or opsonised manner. In the latter case, complement protein C3 can act as an opsonin and complement receptor 1 (CR1), CR3 and CR4 can then facilitate the binding and uptake of the bacteria by the host macrophage (Hirsch et al., 1994). *M. tuberculosis* also utilizes the classical pathway for activation of complement by directly binding to C2a even without the presence of C4b. Subsequently C3b is generated which acts as an opsonin and binds to CR1 (Schorey et al., 1997).

Alternatively *M. tuberculosis* can directly bind to CR3 and CR4 (Zaffran and Ellner, 1997) and get endocytosed in a non-opsonized manner. The non-opsonin-mediated phagocytosis of *M .tuberculosis* by macrophages is best characterized through mannose receptor (MR) (Schlesinger, 1996). A structurally related group of proteins called collectins also facilitate the binding of *M. tuberculosis* to macrophages. They include surfactant proteins, mannose-binding lectins and C1q (Ernst,1998). Besides phagocytosis, an effective host response also requires specific recognition of *M. tuberculosis* or mycobacterial products. Different antigens of *M. tuberculosis* are recognized by various recently identified pattern recognition receptors present on the surface of phagocytic cells. In this regard, lipoarabinomannan (LAM) acts similar to gram-negative lipopolysaccharide (LPS) and promotes immune recognition (Underhill, 1999). This recognition is facilitated by Toll-like receptors which are a group of phylogenetically conserved mediators and are crucial for microbial recognition by macrophages and DCs (Visintin et al., 2001). The TLRs are transmembrane proteins having leucine-rich motifs in extracellular domains like the other pattern-recognizing proteins of immune system. TLR is similar to IL-1 receptor (IL-1R) signaling domain and links IL-1R-associated kinase (IRAK) which is a serine kinase that activates NF-kB like transcription factor to signal the production of cytokines. In context of CD14, TLR2 and TLR6 heterodimer binds to 19-kDa *M. tuberculosis* lipoprotein, TLR4 to heat-labile cell-associated factor and TLR9 to *M. tuberculosis* DNA (Akira et al., 2003).

Innate immunity also facilitates the initiation of adaptive immunity. Antigen presentation of mycobacterial antigens by macrophages and dendritic cells is one of the very

important aspects in that regard. MHC class II which is present on antigen presenting cells present the mycobacterial antigens to CD4+ T cells after processing of these antigens in the phagolysosomal compartment. MHC class I on the other hand is present on all the nucleated cells and present mycobacterial peptides to CD8+ T cells. Studies have confirmed the importance of MHC class I mediated antigen presentation both in animal (Sousa et al., 2000) and human models (Geluk et al., 2000). Nonpolymorphic MHC class I-mediated antigen presentation of mycobacterial lipoproteins by molecules like type I CD1 (-a, -b and –c) expressed on macrophages and DCs is also seen.

Type I cytokines like IL-12, IL-18 and IL-23 produced by macrophages and DCs are important mechanisms to stimulate T lymphocytes (Oppmann et al., 2000). Mutations in the gene coding for IL-12p40 (Altare et al., 1998), IL-12Rβ1 (De Jong et al., 1998), IFN-γ receptor 1 (Holland et al., 1998) and IFN-γ receptor 2 (Dorman and Holland, 1998) have been known to make the patient susceptible to recurrent or fatal nontuberculous mycobacterial infections. In addition to these IL-1 (Dinarello, 1996) and TNF-α (Tsenova et al., 1999) also released by activated macrophages and DCs are known to regulate T cell stimulation.

1.2.2 Acquired immunity

Acquired immunity constitutes both cell mediated (T cell mediated) as well as humoral immunity (B cell mediated). Both T cells and B cells collaborate to fight against any infection. The T cells act by promoting the killing of pathogen infected cells by apoptosis or by cytokine activation of other immune cells while B cells make antibodies to neutralize the pathogen and also target them for destruction (Moore et al., 2001).

Humoral immune response: Role of antibodies in defense against *M. tuberculosis,* an intracellular pathogen!

Since *M. tuberculosis* is an intracellular pathogen, humoral immunity was postulated not to provide any significant protective immune response to the disease. However, immunologists such as Albert Calmette and Elie Mechnikoff emphasized on elevated

cellular immunity as a major defense to tuberculosis in the twentieth century. Since then studies carried out were predominantly focused on cell mediated immune response to tuberculosis. Contrary to the preconceived notion of humoral immunity being ineffective in rendering any significant protection against tuberculosis recent studies have now highlighted the role of B cells in tuberculosis immunity.

B-cells have also been known to modulate the immune response to tuberculosis by antigen-presentation. Inspite of the fact that B-cell mediated antigen presentation is dependent on antigen and immunological conditions, studies have been carried out to utilize B lymphocytes to present antigen to T cells by specific vaccination strategies. In fact, Andersen et al., (2007) in their study demonstrated an effective boost in the BCG primed immunity against *M. tuberculosis* using one such B-cell-targeting vaccine. B cells have also been known to act on the co-stimulatory molecules (B7 and CD40) of other antigen-presenting cells and influence the stimulation of T cell response in a more indirect manner (Radhakrishnan et al., 2003). This is also supported by the study of Radhakrishnan et al. (2003) where they observed that naturally occurring human IgM antibody binds to costimulatory B7 molecule on DCs and potentiate the stimulation of T cells by DCs. They pulsed the DC with Ag and treated them with human IgM Ab in vitro and observed a potent T cell response upon the adoptive transfer of these Ab-treated Ag-pulsed DC.

The question that still lingers is whether the antibodies are protective against *M. tuberculosis* and how can B cells modulate immune response against *M. tuberculosis*? Studies using mAb against mycobacterial arabinomannan, heparin-binding hemagglutinin and 16kDa α crystalline in mouse model have been shown to be efficacious (Reljic et al, 2006). These antibodies were known to act in various manners like diminishing mycobacterial burden or decreasing inflammatory progression and thereby enhancing animal survival (Glatman-Freedman, 2006).. However, in order to use humoral immunity for developing effective vaccines studies have to be carried out to correlate it with protection in human and animal models.

It was also observed that in immunosuppressive phenotypes absence of B cells diminishes the optimal containment of infection in initial stages and the inflammatory progression of TB to chronic stages is delayed. This phenomenon was explained by phase specific function of B-cells. In case of acute infection, B cells are required for effective immunity against pulmonary infection with *M. tuberculosis* and also for generating granulomatous response. However, during chronic phase of infection when the bacilli are dormant and contained, the immunologically active B-cells can act as APC and stimulate T cells, thereby prevent reactivation of disease (Tsai et al., 2006). Abraham et al. (2013) demonstrated that the strong B cell immune response generated by Rv0265 (tuberculosis PPE protein) could be used not only to differentiate tuberculosis patients from BCG vaccinated controls but also detect extrapulmonary and smear-negative pulmonary cases efficiently. Studies have to be conducted in future to further optimize and take advantage of humoral immunity in tuberculosis protection and diagnositics.

Cell mediated immunity : Role in protection and pathogenesis

The fate of tuberculosis infection is therefore mainly governed by the interaction of macrophages with T cells (Kaufmann, 1998). The crucial role of T lymphocytes in immunity to tuberculosis came into light with the emergence of HIV/AIDS epidemic. Tuberculosis became the most common infection following HIV infection. The T cell mediated immunity is mostly governed by CD4+ T helper cells and CD8+ T cytotoxic cells.

1.2.2.1 CD4+ T helper cells

M. tuberculosis replicates within the macrophages, hence MHC class II recognition of pathogen by CD4+ (helper T cells) cells is a crucial step in generation of immune response. Helper T cells can be divided into two phenotypes (Th1 & Th2) on the basis of cytokines they secrete (Mosmann and Coffman, 1989). The Th1 phenotype secrete pro-inflammatory cytokine IFNγ and IL-2 whereas Th2 phenotype secret anti-inflammatory cytokine IL-4, IL-5 and IL-10. Th1 and Th2 are mutually inhibitory to each other. Condos et al. (1998) in their study made an important observation of the dominance of

IFN-γ Th1 response in bronchoalveolar lavage of patients with milder disease showing lack of cavity formation and low bacterial burden. In contrast, in sputum smear positive patients having cavitary lesions was marked absence of Th1 response. The role of Th1 response in protective immune response to tuberculosis was therefore highlighted.

T helper cells mediate the activation of macrophages and other cell mediated reactions including cytotoxic and delayed type of hypersensitive response (Mortaz et al., 2012). The CD4+ T DTH mediates the activation of macrophages and contributes to the killing of the bacilli within the tubercle (Gideon and Flynn et al., 2011). Studies have emphasized the importance of CD4+ T cells in early response to *M. tuberculosis.* This observation was supported by a study carried out by Caruso et al. (1999) on the immune response of CD4+ T cell-deficient aerosol infected mice. They observed that these mice have transient deficiency of IFN-γ in the lungs. Although this deficiency was compensated by CD8+ T cells within four weeks, the mice still succumbed to infection.

1.2.2.2 CD8+ cytotoxic T cells

Another T cell subset, CD8+ T cells, contributes significantly to protection against the disease by lysing the infected cell and inducing apoptosis of the target cells (Schluger and Rom, 1998; Lazarevic and Flynn, 2002). In addition to production of Th1 cytokine IFN-γ, CD8+ T cells restrict the tuberculosis infection by cytotoxicity due to Granule-dependent exocytosis pathway. CD8+ T cells, on recognition of the infected cells release perforin-containing granules. These granules polymerizes the cell membrane of the target cells, thus allowing the entry of effector molecules like granzyme A and granzyme B (serine proteases) and lysing the target cell. CD8+ T cells also exert cytotoxic effect through Fas/ Fas ligand-mediated cytotoxicity. The Fas ligand expressed on the surface of activated CD8+ T cells is cross-linked to Fas receptor expressed on the target cell leading to recruitment of Fas-associated death domain and activation of caspase 8 and

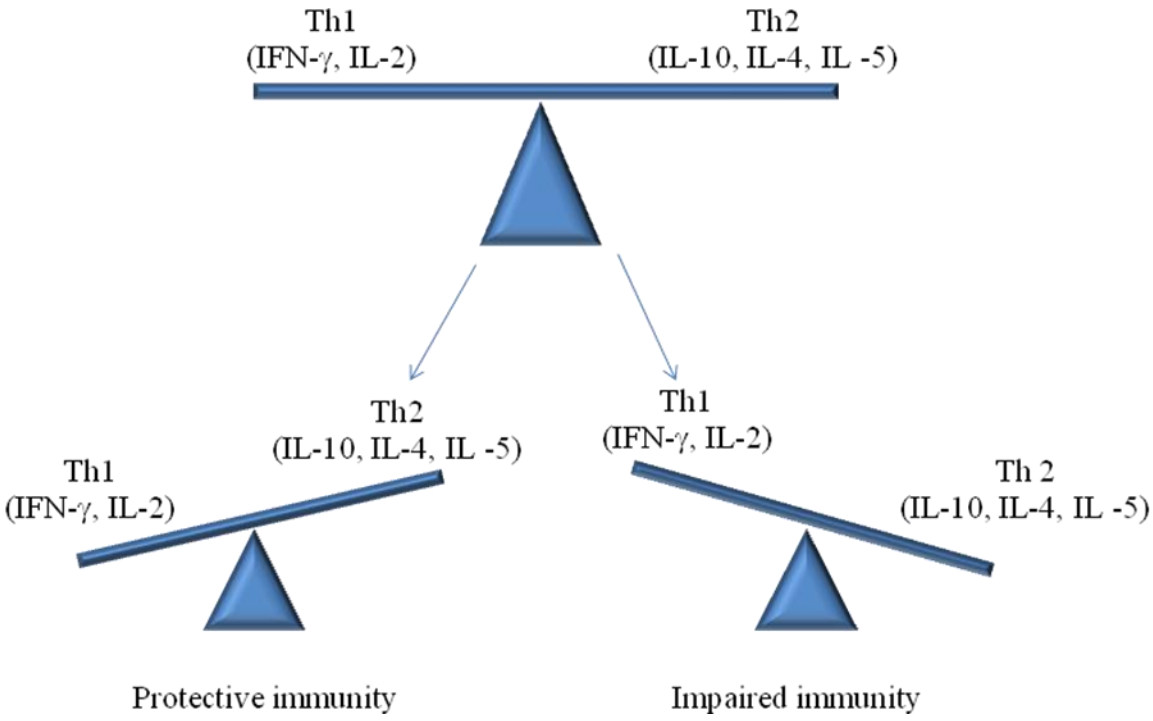


Figure 2 The Th1/Th2 paradium. The homeostasis between Th1 and Th2 cytokines is maintained in the body. Following tuberculosis infection, generation of early Th1 cytokine response helps to contain the infection leading to protective immunity. In case the individual is immunocompromised the Th2 response predominates over the inadequate Th1 response leading to impaired immunity and immunopathology.

finally leading to apoptosis of target cells. Due to lysis of macrophages that are infected with the tubercle bacilli the pathogen is released into the extracelluar environment where it can be taken up by active macrophages that are better equipped for bactericidal action.

CD8+ T cells granules also contain granulysin which is a molecule that has direct microbicidal effect on the intracellular bacteria (Lazarevic and Flynn, 2002). This antimicrobial peptide however, is not found in mouse but is present in humans and mediates the process of killing intracellular mycobacteria The studies on CD4+ T cell KO mice indicate that the cytotoxic potential of CD8+ T cells is dependent on CD4+ T cells and that the susceptibility of these mice to tuberculosis infection might be partially due to impaired CTL response. However, cytokine production remains a crucial mechanism of both CD4+ and CD8+ T cells to generate immune response to tuberculosis (Lazarevic and Flynn, 2002). Scanga et al. (2000) in their study on reactivation of *M. tuberculosis* infection in mice via antibody depletion of CD4+ T cells observed a marked increase in the IFN-γ and CD8+ T cells numbers indicating that CD8+ T cells can efficiently compensate for the IFN-γ levels in such mice.

While it is evident that CD4+ T cells are essential for protective immunity to tuberculosis in the early stages of infection, the critical contribution CD8+ T cells in the later stages of infection has also been reported. Although there is little evidence in vivo, in vitro studies have shown that *M. tuberculosis* might take shelter in epithelial cells which are class II MHC negative as a survival stategy to escape antigen presentation to CD4+ T cells. In such cases the immune response by CD8+ T cells can come into play during this stage of latency (Tully et al., 2005). Besides providing protection in the above mentioned manner researchers have also proposed the possibility of unique functions of CD8+ T cells in immune response to tuberculosis infection. The endogenous antigen presentation to CD8+ T cells via MHC-I restricted manner ensures that CD8+ T cells preferentially recognize and lyse the infected cells, unlike CD4+ T cells that may recognize the infected T cells as well as those cells that have phagocytosed the dead bacteria and their antigen (Lewinsohn et al., 2003).

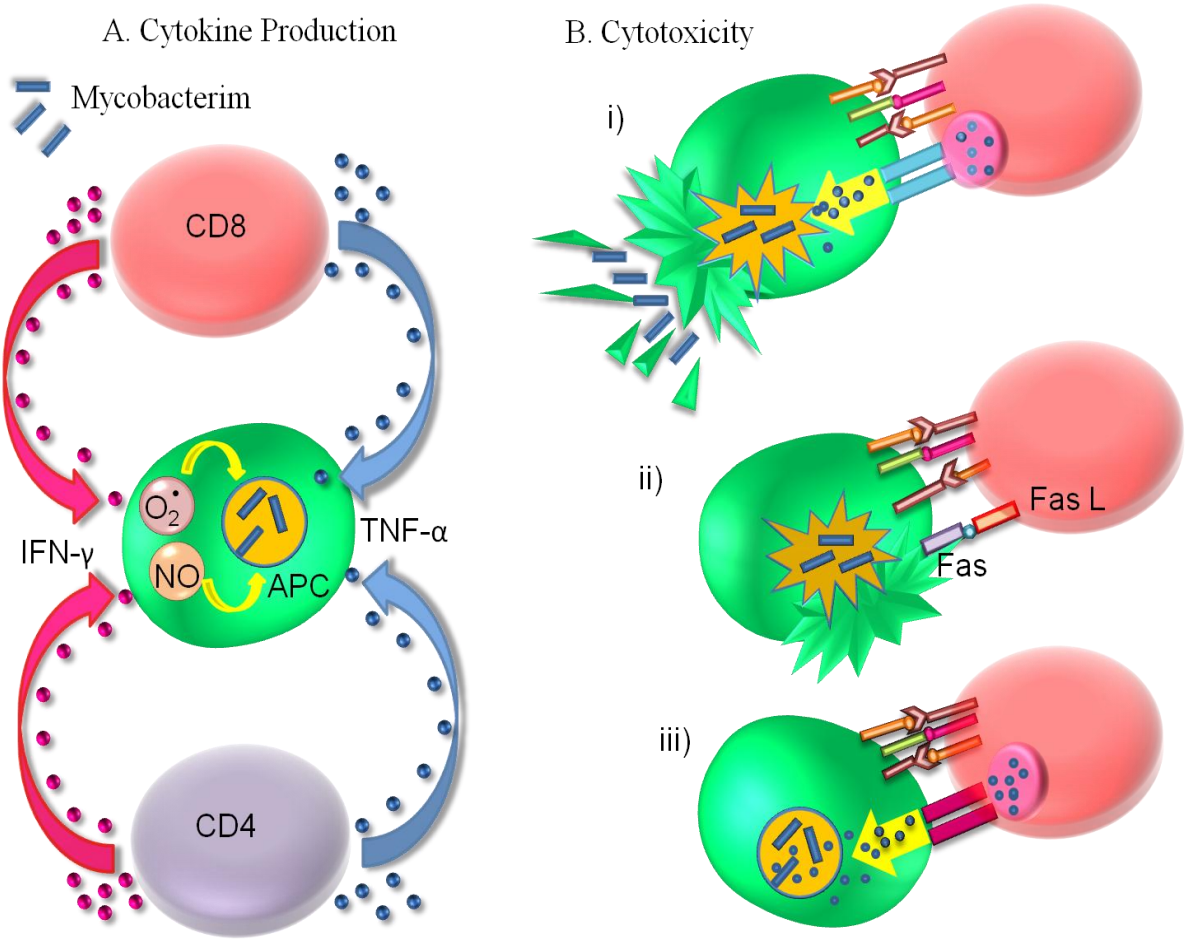


Figure 3 Control of *M. tuberculosis* infection. A) Cyokine Production: Both CD8+ and CD4+ T cells are capable of producing IFN-γ and TNF-α which in turn activates the macrophages to produce reactive oxygen and nitrogen intermediates that mediate the killing of the bacilli **B) Cytotoxicity:** The CD8+ T cells can directly act on the infected macrophages via *i) perforin-containing granules* which polymerize on the cell memberane and facilitates the entry of granzyme A and granzyme B which lyse the targe cells *ii) Fas/ Fas L apoptotic pathway* mediated by Fas receptor on the macrophage that bind to Fas L on the CD8+ T cells leading to apoptosis by activation of caspase 8. *iii) Granulysin* is an antimicrobial peptide that act directly on the intracellular bacteria

1.3 ROLE OF CYTOKINES IN TUBERCULOSIS : A BALANCING ACT

While the effectors in case of a B cell response is the soluble immunoglobulin molecules, which in case of a T cell response are primarily cytokines and chemokines. Cytokines play a key role in orchestrating the immune response. Cytokines may have a protective role, or exacerbate any disease pathology, depending on the type of cytokine involved.

Listeria monocytogenes and *Salmonella* manage to escape the macrophages and infect hepatocytes. The activation of these hepatocytes by cytokines, IFN-γ and TNF-α along with the infiltration of granulocytes is essential host defense against such infections (Langermans et al., 1994). During Toxoplasmosis it has been observed that Th1/Th17 response can aggrevate the hypersensitivity whereas IL-10, TGF-β and IL-27 can counteract the inflammation and prevent immunopathology (Jung et al., 2012). IFN-γ/GM-CSF are being explored as a treatment which can help to improve antigen presentation and immune response against invasive fungal infections like that of Candida and Aspergillus (Mueller-Loebnitz et al, 2013). Similarly in tuberculosis also, cytokines play a major role since the disease primarily involves the T cells of the immune system.

Studies have suggested that the dynamic changes in pro- and anti- inflammatory cytokines governs the outcome of tuberculosis infection (Sahiratmadja et al., 2007; Bose and Jha, 2012). Though studies have emphasized the protective nature of proinflammatory cytokines against the disease, the anti-inflammatory cytokines prevents excessive tissue damage due to inflammation.

1.3.1 Proinflammatory cytokines

1.3.1.1 Interleukin 2 (IL-2)

T cell activation through its antigen receptor is immediately followed by de novo synthesis of IL-2 and IL-2 receptor expression which ensures selective expansion of antigen-specific effector T cell population (Lenardo et al, 1999). The proliferation of CD4+ and CD8+ T cells is thereby the major function of IL-2. The proliferation occurs via proto-oncogenes c-my and c-fos in addition to anti-apoptotic protein bcl-2. Bcl-2 is

involved in glycolysis and cellular metabolism which enhance long-term cell survival (Frauwirth and Thompson, 2004).

IL-2 is also reported to be an important cytokine for cell mediated immune response and granuloma formation (Borish et al., 2003). Studies have suggested that LTBI and active tuberculosis patients can be differentiated by combining IL-2 ELISPOT assay with IGRA. The LTBI secrete IL-2 more than active tuberculosis patients following antigen stimulation (Biselli et al., 2010). This implies that the low bacterial/antigen load may be responsible for increased number of IL-2 secreting and IL-2/ IFN-γ secreting central memory T cells and decreased IFN-γ producing effector cells in LTBI individuals (Millington et al., 2011). Suter-Riniker et al. (2011) in their study demonstrated that increase in IL-2/ IFN-γ ratios may be considered as a biomarker for elimination of *M. tuberculosis* infection. Recently Lindenstøm et al., (2013) emphasised on the importance of the presence of CD4+ KLRG1- non-terminally differentiated IL-2-secreting central memory T cells at the infection site to maintain the population of TNF-α or TNF-α/ IFN-γ coexpressing population of T cells to control bacterial growth. These IL-2+ CD4+ T cells therefore have the potential to replenish T cells and prevent their attrition and functional exhaustion. Zhang et al. (2012) in their study suggested the use of IL-2 and GM-CSF in treatment of multidrug-resistant *M. tuberculosis*.

1.3.1.2 Interferon-γ (IFN-γ)

IFN-γ is a very important component of innate and acquired immunity to tuberculosis and is a potent activator of macrophages. The tubercle bacilli mainly reside in the macrophages but studies reveal that they are also phagocytosed by dentritic cells (Bodnar et al., 2001; Gonzalez-Juarrero and Orme 2001). It was also observed that macrophages preferentially secrete IL-18 whereas dendritic cells secrete IL-12 to stimulate the T cells to produce IFN-γ (Giacomini et al., 2001). A positive feedback loop is created as IFN-γ augments IL-12 production, which in turn induces IFN-γ production.

The protective role of IFN-γ in cell-mediated immunity to tuberculosis has been well established (Flynn et al., 1993). IFN-γ also stimulates the expression of many proteins

involved in antigen presentation including MHC class I and II (Zhou et al., 2009; Giroux et al., 2003). It diverts the differentiation of CD4+ T cells to Th1 lineage and also inhibits Th2 cell proliferation (Smeltz et al., 2002).

IFN-γ, besides being a potent activator of macrophages, enhances the microbicidal activities. It induces the production of reactive oxygen intermediates (ROIs) and nitric oxide (NOIs) (Borish and Steinke, 2003). Studies based on genetic variation in immune response affecting mycobacterial infection suggest that children with mutations in IFN-gammaR gene lead to absence of receptors on macrophage cell surface. These macrophages are unresponsive to IFN-γ stimulation and thereby leaving a defect in TNF-α production. Children having such mutations are therefore more prone to tuberculosis infection (Newport et al., 1996). It was also observed that IFN-γ gene-disrupted mice are unable to control sublethal dose of *M. tuberculosis* administered intravenously or in aerosol from leading to progressive and widespread tissue destruction, necrosis and ultimately disseminated tuberculosis infection (Cooper et al., 1993).

Hence many studies have been focused on *M. tuberculosis* specific IFN-γ production for vaccine. It was observed that PPD and *M. tuberculosis*-culture filtrate could induce IFN-γ production in healthy skin test positive and not in skin test negative healthy individual (van Crevel et al., 1999). However it was observed that the *M. tuberculosis* infected monocytes were capable of stimulating the lymphocytes of both PPD-positive and PPD-negative individuals to produce IFN-γ (Johnson and McMurray, 1994). IFN-γ production can therefore also be used as a marker for identifying latent tuberculosis infected subjects (Dyrhol-Riise et al., 2010). The test most commonly used to identify LTBI is TST skin test. However, the interpretation of TST test is subjective and can lead to incorrect diagnosis. Also false positive results are frequent in individuals who had BCG vaccination in the past or exposure to environmental mycobacteria which share common antigens with *M. tuberculosis*. Also false negative results can be obtained in case the individual is immunocompromised for e.g. HIV (Diel et al., 2010). While interferon gamma release assay (IGRA) has now been developed to overcome these limitations of TST skin test however; the utility of IGRA in countries with high incidence of TB and/or

HIV is still not known (Dyrhol-Riise, 2010; WHO report 2012). It is therefore of utmost importance to screen for immunodominant antigens/epitopes that would differentiate the active tuberculosis patients from LTBI and healthy controls.

1.3.1.3 Tumor necrosis factor – α (TNF-α)

Another Th1 cytokine which contributes significantly to immune response against tuberculosis is tumor necrosis factor – alpha (TNF-α). Mycobacteria or mycobacterial products induce the production of TNF-α by monocytes, macrophages, dendritic cells as well as T cells. In a recent study done by Allie et al. (2013), it was observed that TNF-α inactivation from both myeloid and T- cell sources rendered the mice susceptible to tuberculosis infection. They also reported in their study that while TNF-α production from myeloid cells was crucial in early stages of infection, the T-cell derived TNF-α imparts protection during chronic tuberculosis infection.

The role of TNF-α in maintenance of granuloma was studied by Jacobs et al. (2000). TNF-α is known to increase NFκB expression and thereby increasing the expression of chemokines like IL-8, GROalpha and ENA-78 (Ciesielski et al., 2002). It interacts with the extracellular matrix to direct T cell migration (Czermak et al., 1999).TNF-α neutralization led to decreased chemokine expression (Algood et al., 2004) which could in turn be associated with decreased ability of cells to remain in close proximity or migrate to or aggregate to form granulomas. TNF-α upregulates the expression of adhesion molecules like ICAM-1, VCAM-1 and E-selectin thus facilitating the tethering and diapedesis of leucocytes through the endotheilium into the infected tissue. (Madge et al., 2001). Murine studies have shown that in TNF-/- mice the cell recruitment to the site of infection is delayed although it reached similar levels to those in the wild type infected mice eventually (Roach et al., 2002). This indicates that early cell migration depends on TNF-α. It also synergises with IFN-γ to activate antimycobacterial properties of macrophages thereby up-regulating reactive oxygen and nitrogen intermediates, increases NFκB expression and contributes to the recruitment, migration and retention of inflammatory cells at the site of infection (Roach et al., 2002).

Nevertheless, it was also observed that very high levels of TNF-α can lead to tissue damage and increase immunopathology of tuberculosis. TNF-α can be toxic to epithelial cells as it reduces the amount of surfactant protein produced by type II alveolar cells. Further, it also enhances fibroblast activity and increases the production of fibroblast collagenase (Brenner et al, 1989; Solis-Herruzo et al., 1988). The enhanced production of ROIs also contributes to tissue damage (Solis-Herruzo et al., 1988). TNF-α may attribute to pathological signs and symptoms of *M. tuberculosis* infection like fever, weight loss, anorexia and tissue damage (Mootoo et al., 2009). *M. tuberculosis* makes the infected cells sensitive to both protective effects of TNF-α as well as its toxicity. TNF-α also enhances the cellular toxicity of *M. tuberculosis* (Filley et al, 1991; Filley et al, 1992; Rook et al, 1996). Hence TNF-α, is rightly labeled as 'A cytokine with a Split Personality' by Mootoo et al., owing to its pro- as well as anti- inflammatory attributes.

Several studies have acknowledged the presence of TNF-α production at disease site (Law et al, 1996; Casarini et al., 1999; Dlugovitzky et al., 1999). However, the systemic spill over of TNF-α may lead to unwanted inflammatory effects like fever and wasting. It was observed that clinical deterioration of the disease can be correlated with selective increase (Bekker et al., 1998) and effective anti-tuberculosis treatment with the rapid decrease in plasma levels of TNF-α (Hsieh et al.,1999).

1.3.2 Anti inflammatory cytokine

1.3.2.1 IL-10

IL-10 is a potent Th2 cytokine that inhibits activated macrophages and dendritic cells. It is produced by macrophages after binding of Lipoarabinomannan (LAM) (Shaw et al., 2000) and also shown to be produced following interation of PPE proteins with TLR2 (Nair et al., 2009). IL-10 is also produced by T lymphocytes especially *M. tuberculosis* reactive T cells (Boussiotis et al., 2000).

IL-10 downregulates IL-12 production by *M. tuberculosis* infected macrophages. Hickman et al. (2002) in their study performed the cytokine profiling of infected macrophages and observed downregulation of cytokine IL-12. However when the effects of IL-10 were

neutralized by IFN-γ priming, IL-12 production was resumed. Furthermore, it antagonizes the proinflammatory cytokine response by not only downregulating the production of IFN-γ and TNF-α but also blocks the production of ROIs and NOIs which are essential for *M. tuberculosis* control by interfereing with the intracellular signaling cascade, involving suppressor of cytokine signaling-3 (SOCS3; Cassatella et al., 1999). Giacomini et al. (2006) in their study on *M. tuberculosis* extracytoplasmic factor σE mutants (s*igE*), demonstrated the IL-10 impaired CXCL10 production in sigE mutant-infected DC. However neutralization of IL-10 restored CXCL10 secretion. IL-10 is also known to block antigen processing and presentation by various antigen presenting cells (APC)

Murine studies have demonstrated that IL-10 has a crucial role in TB reactivation from latent stage as well as in chronic pulmonary tuberculosis (Turner et al., 2002; Beamer et al., 2008). However the anti-inflammatory actions of IL-10 in chronic tuberculosis may in fact be contributing to prevention of further damage to the infected tissue. Studies based on transgenic mice models have demonstrated the role of IL-10 in preventing tissue damage and facilitation of *M. tuberculosis* growth at the same time (Murray et al., 1997). However, in case of IL-10 deficient mice no effect was observed (North, 1998) but when IL-10 receptor blocking antibody was used it as observed that there was protective effect due to absence of IL-10 (Moore et al., 2001). Studies on human population have also correlated the presence of IL-10 with the susceptibility to tuberculosis infection (Boussiotis et al., 2000; de la Barrera et al., 2004). Recent study shows that stalling of phagosome maturation due to IL-10 in human macrophage facilitates *M. tuberculosis* persistence (O'Leary et al., 2010). Thus, the benefits of anti-inflammatory effect of IL-10 on tissue damage are outweighed by its role in reactivation and persistence of *M. tuberculosis*.

1.3.2.2 Transforming growth factor – β (TGF-β)

Transforming growth factor β is mainly secreted by monocytes and macrophages and other cell types like dendritic cells, CD4+ T regulatory cells (Aung et al, 2005). It synergises with the effects of IL-10 and therby modulates the immune response by down-regulating acquired immunity and de-activating macrophages. It is a pluripotent cytokine

capable of generating anti-inflammatory and some proinflammtory effects. The proinflammatory effects include enhancement of chemotaxis of monocytes and increased expression of Fc receptors (Schluger and Rom, 1998).

However, the anti-inflammatory actions of TGF-β are predominant which include promoting immune tolerance and limiting the pathological inflammation in synergy with IL-10 (Chen et al., 2003). TGF-β also suppresses the expression of costimulatory molecules in APCs and down-regulates production of IFN-γ and TNF-α thereby reducing the generation of ROIs and NOIs (Ruscetti et al., 1993) Decreased production of cytokines like IL-12 further contribute to down-reguation of T cell function and proliferation (Gorham bet al., 1998). TGF-β levels have been shown to be up-regulated in granuloma of active tuberculosis patients (Toossi et al., 1995) and high levels of TGF-β have been correlated with severe forms of the disease. Like IL-10, TGF-β also reduces the harmful inflammatory effects by its anti-inflammatory properties.

1.3.2.3 Interleukin-4 (IL-4)

IL-4 stimulates the development of Th2 cells from naïve CD4+ T cells and also induces production of IgE antibodies. IL-4 is in turn secreted by Th2 cells thereby further differentiating and proliferation Th2 cells in an autocrine fashion. It is also produced by activated mast cells and basophils (Borish and Steinke, 2003).

IL-4 exerts its immunosuppressive effect by reducing IFN-γ production by down regulating IL-12 and IL-12 receptor and also antagonizes the effects of IFN-γ leading to deactivation of macrophages (Nakamura et al, 1997). The downregulation of IL-2 by IL-4 further contributes to the Th1 to Th2 switch. Studies have also demonstated the correlation of increased production of IL-4 in human tuberculosis patients with excessive tissue damage and cavitary disease (van Crevel, 2000). Therefore, cytokines of both pro- and anti- inflammatory groups are crucial in maintaining a balance between inflammation and bacterial killing to minimize tissue damage.

1.4 GRANULOMA IN TUBERCULOSIS: BATTLE FIELD OR SHELTER FOR MYCOBACTERIA

"On the basis of my numerous observations I consider it established that, in all tuberculous affections of man and animals, there occur constantly those bacilli which I have designated tubercle bacilli and which are distinguishable from all other microorganisms by characteristic properties." – Robert Koch , 1882.

With these words Koch announced the discovery of the etiologic agent of tuberculosis. The term " tubercle" was given by Sylvius in the year 1650 and refered to apparent lung nodules which was a common feature of "consumption" disease. Today, these tubercles are known as granulomas.

Granuloma is an organized microscopic aggregation of immune cells. It consists of infected macrophages in the center, an inner cuff of mainly resting and activated macrophages which are transformed into epithelium like cells (epithelioid cells), multinucleate giant cells and foamy cells surrounded by an outer cuff of predominantly lymphocytes and other leukocytes like neutrophils, dendritic cells and sometimes fibroblasts. The granuloma often has a necrotic center. (Ramakrishnan et al., 2012)

1.4.1 Types of Granuloma

Granuloma can be divided into two types based on their etiology :

a) Epithelioid granuloma are characterised by the presence of epithelioid cells (modified macrophages) and sharp circumscribed module such as in tuberculous granuloma or sarcoidal granuloma. The tuberculous granuloma most often presents with caseous necrosis whereas sarcoidal granuloma has non caseating center.

b) Histiocytic granuloma characterized by ill defined nodule containing phagocytic histiocytes (tissue macrophages) e.g. Foreign-body granuloma, Rheumatoid granuloma and Rheumatic granuloma

1.4.2 Immunopathology of granuloma:

Once *M. tuberculosis* enters the airways through aerosols, it first encounters macrophages and dendritic cells. Unless killed by alveolar macrophages, the surviving bacilli migrate to the lung parenchyma initiating the inflammatory response. The DC phagocytose the bacilli and drain to the thoracic lymph nodes (LN) thus priming the T cell response (Bhatt et al., 2004). When tubercle bacilli invade and replicate in the alveolar macrophage, the site of primary infection is known as Ghon focus. The draining lymphatics, regional lymph node involved along with Ghon's focus is known as Ghon complex (Vijayan, 2002). This site is primarily and transiently infiltrated by polymorphonuclear neutrophis (PMN) which are later replaced by activated macrophages.The recruitment of inflammatory cells like PMN, macrophages and T and B lymphocytes occur under the influence of chemokines. These chemokines are in turn regulated by cytokines like TNF-α and IFN-γ. The dendritic cells then drain into the lymph nodes and sensitise the CD4+ T helper cells and CD8+ T cytotoxic T cells and initialize the adaptive immune response.The migration of these sensitized T lymphocytes along with other leukocytes like monocytes, dendritic cells and neutrophils give rise to the structure known at granuloma. The activated macrophages in the necrotic center and the dense surrounding of CD4+ and CD8+ T cells forms a cellular wall that restrict the spread of the bacteria (Saunders et al. 2002).

Granuloma therefore appears to be a prerequisite for limiting the infection. It provides a framework whereby the T cells, which produce cytokines, remain in close apposition with the macrophages that become activated and achieve a mycobacteristatic state. It also provides a toxic environment for the mycobacteria in addition to protecting the delicate alveolar tissue in the process (Saunders et al. 2002).

After two to four weeks of infection, additional host responses to *M. tuberculosis* develop in the form of the tissue damaging response and macrophage activating response. The tissue damaging response occurs due to delayed type of hypersensitivity (DTH) reaction to various mycobacterial antigens. Whereas, the macrophage activation reponse is facilitated by activation of macrophages by the cell mediated immune response (CMI)

rendering the macrophages capable of killing and digesting tubercle bacilli (Raviglione and O'Brien, 2012). A strong granulomatous reaction within the first few days is a prerequisite to contain the infection which is associated with the innate immune response. The development of acquired immune response occurs in the form of CMI and DTH. The development of caseous necrotic centre has been associated with DTH. The intense cytokine and cell- cell interaction environment also leads to necrosis of macrophages and epithelioid cells. The material released due to necrosis has 'cheese like' appearance hence it is known as caseaous necrosis (Fayyazi et al., 2000). This is a host defence strategy to destroy its own tissue in order to control the uninhibited intracellular multiplication of tubercle bacilli which would otherwise be detrimental. Majority of bacteria are killed in this process; however those that survive are unable to multiply in the external milieu. Within the caseous tissue, the acidic pH, low availability of oxygen and the presence of toxic fatty acid make the environment hostile for the tubercle bacilli. These anoxic conditions developed by macrophages and T lymphocytes in the granuloma results in inhibition of replication and even killing of mycobacteria.This leads to the formation of typical granulomas in tuberculosis characterised by central caseation known as soft tubercle. Although sometimes there is no caseation, and such granulomas are known as hard tubercle.

At this stage in the presence of a good CMI, the granuloma are surrounded by large number of lymphocytes particularly IFN-γ secreting CD4+ T cells causing the caseating granuloma to shrink as they become fibrotic and then calcified. (Vijayan,2002). In immunodeficient individuals however, the *M. tuberculosis* infection leads to formation of granulomas containg large rich inactivated macrophages, with very few lymphocytes at the periphery (Ulrichs et al., 2005). The absence of protective CMI response and the uncontrolled caseating destructive response leads to tissue destruction. As a result the surrounding tissue is progressively damaged, causing the lesion to enlarge further. The caseous necrotic center of the granuloma cavitates and the bacilli is released into the bronchi. The spread of the bacilli from such cavitary lesions can occur through airways, lymphatics or hematogenous transmission.

1.4.3 Cell death in granuloma

Macrophages in tuberculous granuloma can undergo both necrosis and apoptosis. (Fayyazi et al., 2000). Both mycobacterial and host factors contribute in this process. Caseating granulomas formed from the breakdown of immune cells especially macrophages are characteristic feature of tuberculosis. Histological studies from the pre-chemotherapy era correlated with the presence of caseum in early granuloma with increased bacterial numbers as opposed to noncaseating lesions. The macrophage rich areas shown enhanced bacterial growth and the cellular debris contained bacterium –rich exudates indicating the release of bacteria from necrotic macrophages. These mycobacteria thereby grow more exuberantly following their release into an extracullar mileu due to macrophage necrosis (Algood et al., 2005). The proliferation of bacteria in the excellular environment following necrosis exceeds much more than that due to phagocytosis of apoptotic macrophages (Ramakrishnan, 2012). The extent to which these extracellular bacteria are rephagocytosed by new macrophages is unclear.

Apoptotic macrophages in contrast to necrosis form apoptotic bodies of intact membranes in which the bacteria remain encased. The phagocytosis of apoptotic macrophages containing bacteria by multiple uninfected macrophages provides new niches for bacterial replication and nullifying any bactericidal effects of apoptosis (Ramakrishnan, 2012). However, another notion is that when under the effect of CD8+ T cells the infected inactive macrophages undergo apoptosis and they are taken up by other activated macrophages that are better equipped to kill the tubercle bacilli (Lazarevic and Flynn, 2002).

In comparison to apoptosis, the magnitude of bacterial proliferation promoted by necrosis is much greater. The bacterial proliferation is exteremely high in external milieu as compared to apoptotic death and rephagocytosis. It is also proposed that by shifting the balance from apoptosis to necrosis *M. tuberculosis* reduces the DC- mediated cross-priming of T cells. Apoptosis is also assumed to facilitate bacterial killing and stimulation of T cells by promoting antigen presentation, whereas necrosis leads to release of bacteria thus enhancing inflammation and thereby tissue damage (Philips and Ernst, 2012).

Granuloma is therefore considered to be the structure to 'wall off' the bacteria and therefore preventing the bacilli from dissemination to other sites of the lung or other parts of the body. It facilitates the formation of local milieu for immune cells to communicate with each other by physical contact or via production of cytokines and chemokines. (Aggarwal, 2003). The presence of healed fibrotic and calcified granulomas in healthy individuals signifies that the granuloma tried to restrict the growth of bacteria but failed. It can therefore be implied that in the absence of granuloma, there will be uncontrolled proliferation and dissemination of tubercle bacilli (Ramakrishnan 2012).

1.4.4 Cell population involved in Granulomatous response

1.4.4.1 Neutrophils

Following aerosol infection polymorphonuclear neutrophils (PMN) are among the first cells to arrive within few hours at the site of infection. However, the contribution of these cells in immune response to tuberculosis is unclear. PMN are considered as first line of defense against tuberculosis and rapid migration of PMN to the site of infection is considered as a pre-requisite for focal organization of pulmonary granulomas in tuberculosis (Seiler et al., 2003). Some studies have indicated that PMN exhibit bactericidal effects though NADPH oxidase-dependent mechanisms (Kisich et al., 2002) however other studies have reported that the tubercle bacilli escapes this oxidative killing (Corleis et al., 2012). In fact, other studies suggest that limited ability of neutrophils to restrict mycobacterial growth as compared to macrophages leads to enhanced pathology and that neutrophils may favour the mycobacteria by acting as a "Trojan horse".

They are activated by lipoarabinomannan of *M. tuberculosis* (Riedel et al., 1997). They contribute to the killing of bacteria and facilitate the recruitment of leukocytes through chemokines like MCP-1 and IL-8 leading to initiation of inflammatory response (Riedel et al., 1997; Seiler et al., 2003). Neutrophils do not appear to have a significant role once the granuloma matures however their presence is observed when the granuloma starts to become necrotic. Their role in granuloma necrosis is still not clear.

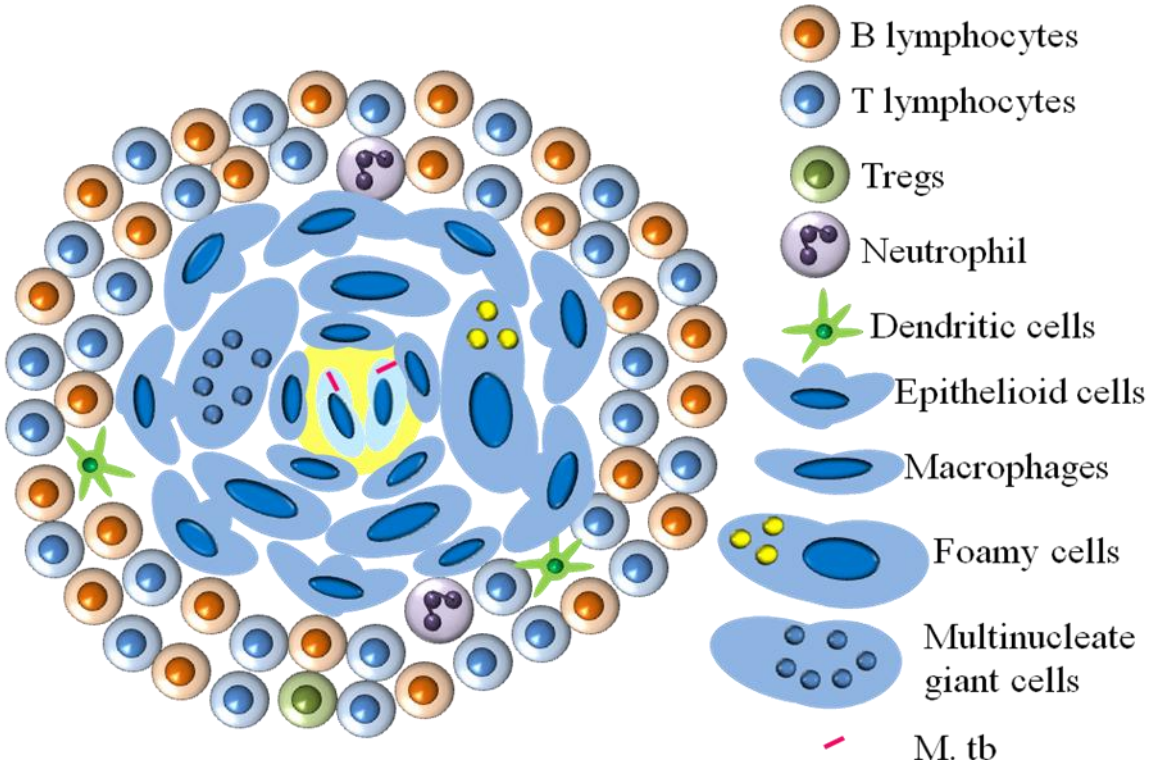
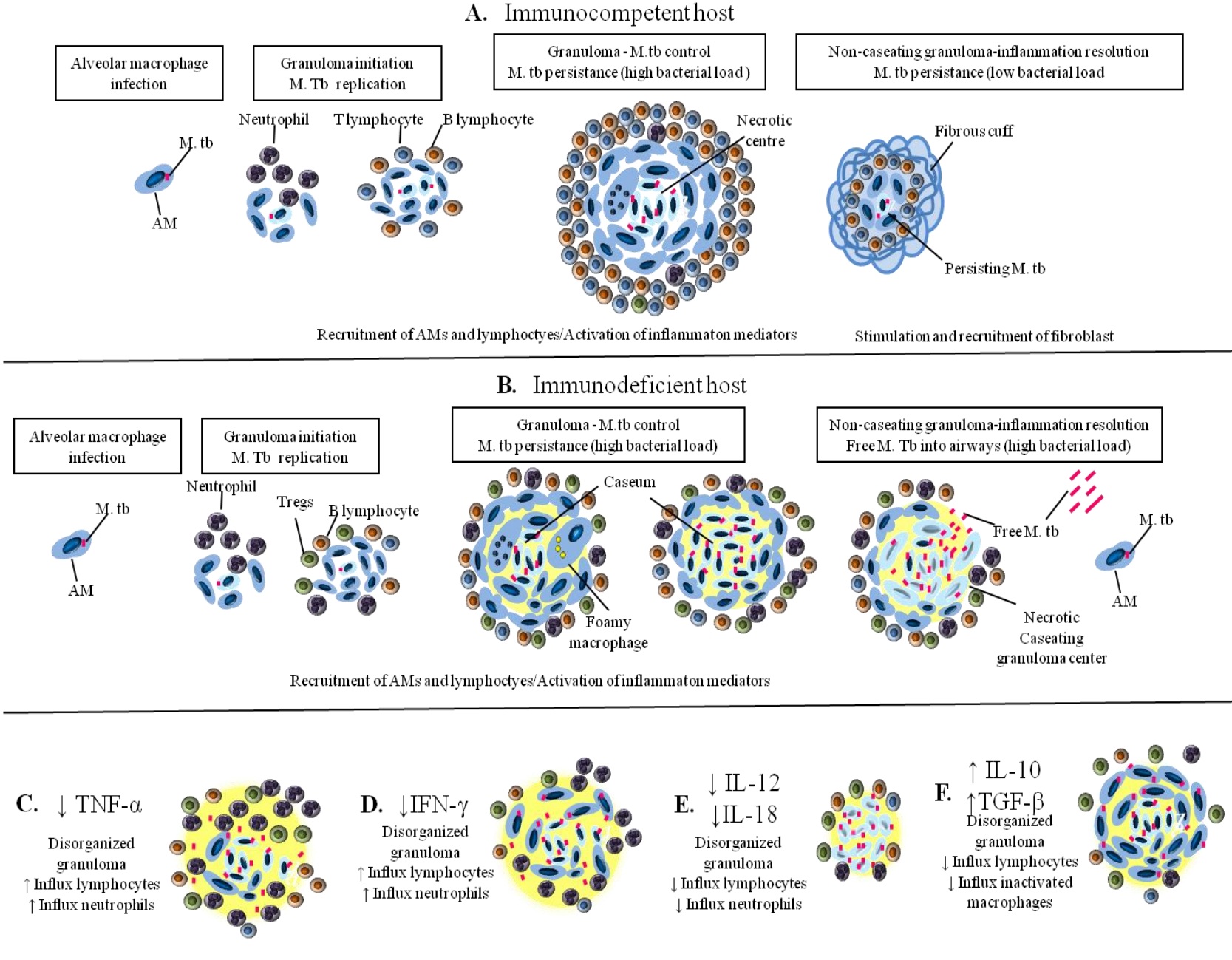


Figure 4: Granuloma: Microscopic aggregate of infected macrophage in the center having caseous necrosis along with other forms of macrophages including epithelioid cells, foamy cells and multinucleate giant cells. The center is surrounded by a collar of leukocytes, mainly, B and T lymphocytes, neutrophils and dendritic cells.


A. Immunocompetent host
Alveolar macrophage infection
Granuloma initiation M. Tb replication
Granuloma - M.tb control M. tb persistance (high bacterial load)
Non-caseating granuloma-inflammation resolution M. tb persistance (low bacterial load
M. tb
AM
Neutrophil
T lymphocyte
B lymphocyte
Necrotic centre
Fibrous cuff
Persisting M. tb
Recruitment of AMs and lymphoctyes/Activation of inflammaton mediators
Stimulation and recruitment of fibroblast
B. Immunodeficient host
Alveolar macrophage infection
Granuloma initiation M. Tb replication
Granuloma - M.tb control M. tb persistance (high bacterial load)
Non-caseating granuloma-inflammation resolution Free M. Tb into airways (high bacterial load)
M. tb
AM
Neutrophil
Tregs
B lymphocyte
Caseum
Foamy macrophage
Free M. tb
M. tb
AM
Necrotic Caseating granuloma center
Recruitment of AMs and lymphoctyes/Activation of inflammaton mediators
C. ↓ TNF-α
Disorganized granuloma
↑ Influx lymphocytes
↑ Influx neutrophils
D. ↓IFN-γ
Disorganized granuloma
↑ Influx lymphocytes
↑ Influx neutrophils
E. ↓ IL-12
↓IL-18
Disorganized granuloma
↓ Influx lymphocytes
↓ Influx neutrophils
F. ↑ IL-10
↑TGF-β
Disorganized granuloma
↓ Influx lymphocytes
↓ Influx inactivated macrophages

1.4.4.2 Monocyte

The infected macrophages attract immune cells including other monocytes to the site of infection by chemokine production. The monocytes can be recruited from the circulation or tissue reservoirs.The role of these newly recruited monocytes in controlling *M. tuberculosis* infection remains undefined. They are either exposed to mycobacterial soluble products or get infected with *M. tuberculosis*. The local production of IFN-γ and TNF-α drives the differentiation and activation of monocytes within the granuloma.

The matrix metalloproteinases (MMPs) secreted by monocytes are known as granuloma creators and tissue destroyers (Salgame, 2011). MMP-9, secreted by monocytes following *M. tuberculosis* stimulation, degrades type IV collagen and facilitates leukocyte extravasation to the site of infection (Goetzl et al., 1996). Studies have demonstrated the role of MMP-9 in recruitment of leukocytes and formation of granuloma to restrict bacterial growth, however excessive MMP-9 may lead to host tissue injury in TB (Price et al., 2003). Volkman et al. (2010) demonstrated that the MMP-9 upregulation in epithelial cells due to secreted 6-kDa early secreted antigenic target (ESAT-6) protein leads to recruitment of newer monocytes and macrophages which is a prerequisite for granuloma maturation.

1.4.4.3 Monocyte-Derived Cells

1.4.4.3.1 Macrophages

Macrophages play a central role in immunity to *M. tuberculosis* but at the same time act as their shelter. The macrophages resort to various inflammatory and non-inflammatory mediators like cytokines and fibrinolytic enzymes to combat the infection. In case of nonpathogenic mycobacterial infection the bacteria are killed by acidification of the phagosomal compartment. *M. tuberculosis* however, prevents the incorporation of ATP/proton pump into the phagosome and restricts the fusion of this vacuole with lysosome.

In case of resting macrophages, *M. tuberculosis* can prevent phaogolysosomal fusion. However, the macrophages activated by IFN-γ can stimulate autophagy and overcome

this block (Deretic et al., 2009).The activated macrophages can also exert the bactericidal effects through antimicrobial peptides, reactive oxygena or nitrogen intermediates. The infected macrophages also respond to TNF-α mediated apoptosis thereby exposing the bacilli to direct antimicrobial effects (Lee et al., 2006). The apoptotic body so formed can be engulfed by newly recruited macrophages and dendritic cells to accelerate the elimination of the bacilli by inducing adaptive immune response (Hinchey et al., 2007).

1.4.4.3.2 Multinucelated cells

Multinucelated Langhans giant cells (MGC) are characteristic feature of tubercular granuloma. These are formed from macrophages that fuse together to form giant cells. Mycobacterial lipoarabinomannan is known to trigger the TLR2-dependent cell activation which eventually leads to fusion process (Puissegur et al., 2007). The coculture of macrophages with activated T cells also induces MGC formation through CD40/ CD40L interation and IFN-γ secretion (Sakai et al., 2011). It was observed that the the poorly virulent strains of mycobacteria could only induce small multinucleate cells (MCs) formation which have limited ability to take up mycobacteria but fail to differentiage into MGC stage. MGCs loose their ability to phagocytose bacteria as they no longer express mannose receptor/CD11b (phagocytic receptors) while they retain their antigen presentation ability. MGCs also display NADPH oxidase acitivity. This implies that MGCs seem to devote themselves in the elimination of bacilli ingested in the previous stages of differentiation i.e. macrophages and multinucleate cells (Lay et al., 2007).

Since the presence of MGC is considered to be hallmark of tuberculosis infection, it would be interesting to know how patient monocytes differ from healthy control monocytes in multinucleate giant cell formation and the cytokines that influence this process.

1.4.4.3.3 Monocyte-Derived Dendritic Cells (mDCs)

mDCs present in granulomas at early stages drain the lymph nodes and educate the T cells. It was observed that large exchange of DCs in the late stages of infection is

indicative of immune surveillance (Schreiber et al., 2011). DCs do not have very efficient microbicidal activity, however, they limit the replication of *M. tuberculosis*. DCs infected with mycobacteria stimulate the T cells but at the same time may also serve as a vehicle for the pathogen to spread in other parts of the body (Tailleux et al., 2003).

1.4.4.3.4 Foamy Macrophages

Human tuberculous granulomas classically contain foamy macrophages. Their foamy appearance is due to lipid bodies formed as a result of accumulation of intracellular lipids. The lipids present in these macrophages are predicted to serve as a source of nutrients for the bacteria. Foamy macrophages are also instrumental in maintaining *M. tuberculosis* in a dormant state. (Peyron et al., 2008). Moreover since they lose their phagocytic and bactericidal activities they have been used as model of dormancy to test the drugs active at this stage.

1.4.4.4 T lymphocytes

1.4.4.4.1 CD4 T cells

The crucial role of CD4+T cells in combating the mycobacterial infection and granuloma formation was demonstrated in knockout mice studies. It was observed that in MHC II $^{-/-}$ and CD4 $^{-/-}$ mice, not only the granuloma formation was delayed by a week but the granuloma that was formed was also disorganized and failed to contain the infection despite the macrophages displaying normal NO synthesis (Caruso et al., 1999). Ordway et al. (2007) in their study on mice models demonstrated strong Th1 response (characterized byIFN-γ and TNF-α production) by the CD4+ T cells and that the removal of CD8+ T cells at this stage did not affect bacterial growth. However, the Th1 response decreased in the later stage of the disease and concomitant removal of CD8+ T cells lead to reactivation and increased bacterial growth. However, it was also observed that CD8+ T cells were unable to compensate for the reduced Th1 expression in CD4+ T cells which implies that CD4+ T cells are curial at all stages of the infection. The presence of CD4+ T cells is also essential for the expression of Th1 like response by CD8+ T cells.

1.4.4.4.2 Th17

In addition to CD4+ and CD8+ T cells Th17 type of cells are also shown to play a role in *M. tuberculosis* infection, IL-17 secreted by Th17 cells helps in recruitment of neutrophils and activating macrophages It also recruits effector Th1 cells to the site of infection. Most often it is the γ/δ T lymphocytes that predominantly secrete IL-17; however in some cases IL-17 secreting CD4+ T cells are also present in the granuloma.

1.4.4.4.3 Regulatory T cells

FoxP3+ regulatory T cells are recruited to granuloma to function as gate keepers of excessive immune response. CD4+CD25+Foxp3+ T regs were also found in the granulomatous lesions of active tuberculosis patient. They are located not only in the primary granuloma but also at the draining mediastinal lymph node where secondary granulomas are found. Studies have suggested the role of Treg cells in establishment of persistant infection. Shafiani et al. (2010) in their study on mice observed theat Treg proliferation takes place when *M. tuberculosis* is transported to the pulomonary lymph node (pLN). These *M. tuberculosis* specific Tregs were responsible for delaying the priming of effector CD4+ and CD8+ Tcells in the pTN and thereby their recruitment to the granuloma. This delay results in high bacterial load in these mice. This implies that *M. tuberculosis*-specific T cells specifically and significantly restrict the protective immune response during tuberculosis infection.

1.4.4.4.4 Cytotoxic T cells

Studies on CD8+ T cells deficient mice (ß2m−/− MHC-I-deficient mice) revealed that these mice were more susceptible to mycobacterial infection than the wild type animals, although the severity of the disease was not more than that of the CD4+ T cell-deficient mice. The CD8+ deficient mice showed increased susceptibility to *M. tuberculosis* irrespective of the inoculum size. (Ladel et al., 1995; Flynn et al., 1992). The CD8+ T cells were found to be localized in the periphery in the early stages of infection but migrate towards the center as the disease progresses (Gonzalez-Juarrero et al., 2001). The outer mantel of activated CD8+ T cells appears to be playing a role in retaining the infection; however it reduces the possibility of contact dependent killing of infected cells

and eradication of infection. CD8+ T cell deficient mice showed granuloma formation, however, these granulomas were functionally impaired and had marked central necrotic zone which was absent in case of wild-type mice. This pathology could be the result of lack of apoptotic induction in infected cells leading to generation of these cells and enhanced neutrophil recruitment to the site of infection (Flynn et al., 1992).

Within the granuloma, the granule mediated killing of the tubercle bacilli is the major defense mechanism used by CD8+ T cells. Rahman et al. (2009) showed that the presence of CD8+ T cells expressing low perforin and granulysin correlated with the elevated levels of Tregs. Another important role of CD8+ T cells was highlighted by Ordway et al. (2007) in which they demonstrated the release of CXCL1 chemokine by CD8+ T cells following aeorosol infection with *M. tuberculosis*. CXCL1 facilitates the chemotaxis of CD4 and CD8 T cells to the infection site.

Hence the studies based on various T cell subsets present in tubercle granuloma indicate that with the establishment of clinical tuberculosis the immune response is skewed towards Th2/Treg suppressive response. This in turn antagonizes the Th1/Th17 protective response and the functionality of CTLs is also compromised. Therefore, strategies for designing new immunotherapies can be directed towads enhancing the cell-mediated immune response by targeting Th2/Treg cells (Rahman et al., 2009).

1.4.4.5 B lymphocytes

The role B cells play in immunity to tuberculosis and granuloma is still unclear. Maglione et al. (2007) in their study on B cell -/- mice observed exacerbated immunopathology following aerosol infection with 100 CFU (colony forming units) of *M. tuberculosis* along with increased influx of neutrophils to the infection site. These mice also showed increased production of IL-10 in the lungs in addition to increased susceptibility to lung infection. Phuah et al. (2012) demonstrated the presence of B cell clusters in granuloma and peripheral node in cynomologus macaques monkey model. They suggested that the presence of *M. tuberculosis* specific B cells secreting antibodies

and expressing elevated CXCR5 chemokine and HLA-DR expression within the granuloma might have a role in modulating local control of infection.

1.4.5 The key players of Granuloma: Cytokines

Interaction of *M. tuberculosis* with alveolar macrophages and DC leads to the production of IL-12 and IL-23 by these cells. These cytokines in turn prime the Th1 cells that play a crucial role in granuloma assembly and immune response. The activated Th1 cells release IFN-γ and IL-2. In an immunocompetent individual the granuloma is characterised by the presence of IFN-γ producing CD4+ T cells. Sugawara et al. (1998) in their study based on IFN-γ-gene- disrupted mice showed granuloma formation only by avirulent strains but not by virulent strains of *M. tuberculosis*. They observed that when these mice were subjected to infection by BCG Pasteur and H37Ra strains they induced granuloma in spleen liver and lungs. These granulomas were infiltrated by macrophages and multinucleate giant cells but lacked necrosis. However when the mice were infected with H37Rv strains (virulent strain), disseminated abscesses were formed due to failure to induce granuloma in various organs. This study therefore substantiated the importance of IFN-γ in macrophage activation and granuloma formation mechanism.

Another proinflammatory cytokine that is particularly important in this regard is TNF-α. TNF-α is known to be crucial in maintainance of granuloma. Bekker et al. (2001) in their TNF-α knockout (KO) mice demonstrated the logarithmic growth of intracellular bacilli following BCG infection. However infection with BCG-secreting murine TNF lead to bacterial killing which was in turn associated with high inducible NO synthase (iNOS) production. Also in the same study they obsevered that iNOS-KO macrophages could only kill the bacteria after BCG-TNF infection. These results suggest that the bacterial killing by the macrophages takes place in two ways: 1) TNF-α- dependent- iNOS dependent manner and 2) TNF-α- independent- iNOS dependent manner. Another TNF-α gene disruption study in $TNF^{-/-}$ mice demonstated widespread dissemination of *M. tuberculosis* following aerosol infection and poorly formed granulomas with extensive necrosis and neutrophilic infiltration of the alveoli (Bean et al., 1999). These studies

therefore highlight the role of TNF-α in organized granuloma formation and killing of the tubercle bacilli.

In case of chronic infection apart from TNF-α and IFN-γ , IL-2 also plays a crucial role. Lindenstrøm et al. (2013) in their study observed that *M. tuberculosis* infection of BCG immunized mice was controlled up to 7 weeks of infection following which, the protection loss was correlated with the disappearance of IL-2+CD4+T cells. However, a booster dose of subunit vaccine (Ag 85B-ESAT-6 + CAF01) was able to induce the expansion of IL-2+CD4+T cells coexpressing TNF-α or TNF-α/ IFN-γ which was maintained and thus controlled the bacterial growth in the late stage of infection. It was observed that the central memory cell so produced could replenish the T cells at the site of infection and prevent functional exhaustion even when the animals were challenged two weeks post infection. However, variable results have been obtained regarding the role of IL-2 in multinucleate giant cell formation (Birkness et al., 2007; Gasser and Most,1999).

The other group of cytokines that regulates the immune response in granuloma is the anti-inflammatory cytokines for e.g. IL-10. Murray et al. (1997) in their study on transgenic mice observed that although on one hand IL-10 prevents excessive tissue damage by reducing inflammation, on the other hand it contributes to the mycobacterial growth. De la Barrera et al. (2004) demonstrated increased IL-10 production in tuberculosis patient than healthy controls. The IL-10 production also affected the lytic property of T cells. However when IL-10 was neutralized with antibody or by adding exogenous IFN-γ, it led to increased lytic activity of both CD8+ and CD4+ T cells. Thus, it appears that the beneficial effect of IL-10 is overridden by its contribution to mycobacerial persistence inside host macrophages and reactivation of tuberculosis. IL-10 depletion decreases bacterial burdens and increases the influx of IFNγ-producing T cells to the site of lung infection **(**Redford et al., 2010)

The functions of IL-10 are further synergized by the action of TGF-β. Like IL-10, it also reduces the tissue damage as a result of pathological inflammation but at the same time suppress APCs costimulatory molecules, iNOS production and downregulates T cell

proliferation and cytokine production. Toossi et al. (1995) in their study demonstrated upregulated TGF-β in monocytes and macrophages in granuloma of active tuberculosis patients and high levels of TGF-β correlated with severe stages of infection.

Another molecule with which IL-10 synergizes is IL-4 and inhibits macrophage cytotoxic activity (Oswald et al., 1992). IL-4 is not only predominantly produced by Th2 lympocytes but also induces Th2 differentiation. Both IL-4 and IL-10 Th2 cytokines contribute significantly in inhibiting Th1 development and activation in addition dampening macrophage activation and bactericidal activity. Animal model studies have associated the increased production of IL-4 with cavitary tuberculosis (van Crevel et al., 2000). IL-4 has also been known to contribute significantly to TNF-α toxicity (Hernandez-Pando et al., 2004). In this study it was observed that IL-4 deficient mice not only have significantly low bacterial load but also reduced TNF-α toxicity following TNF-α challenge.

1.5 SURVIVAL OF MYCOBACTERIA: DITCHING THE HOST IMMUNE RESPONSE

Despite various immune cells and responses, mycobacteria manage to escape the host immunity, survive, proliferate and spread infection. It was observed that mycobacteria resist acidification of the macrophage phagosome to pH 6.4 thus preventing the environment from being hostile for its survival and fail to fuse with lysosome (Deretic et al., 2006; Russel et al 2011) . Cell-wall lipids and other mycobacterial effectors bring about the modulation of phagosome. It was observed that the mutants were partially defective in phagosome modulation which resulted in pH5.8 of the residing vacule of mycobacteria thus arresting its growth. Additionally, on activation the macrophages overcome the effect of mycobacteria on phagosome maturation and make it more acidic at pH5.2, which is a bactericidal environment.

As described earlier the development of granuloma is to facilitate the interaction of immune cells and generation of robust cellular immune response against the bacterial antigens. However, the mycobacteria manage to manipulate the response in its favour. *M.*

tuberculosis releases cell wall components lipoarabinomanan and arabinomannan inside the infected cells and accumulate in multilamellar bodies which contain both bacterial and host components. The multilamellar bodies then unite with microvesicle lysosome known as MHC class II-enriched compartment. This vescicle then escapes the infected macrophage by exocytosis and released into external milieu as exosome. The exosomes are then internalized by the neighboring cells. The mycobacterial cell wall proteins in the exosomes released from infected macrophages were identified as belonging to Ag85 family.

1.6 ROLE OF HLA IN CELL MEDIATED IMMUNITY

The two main reasons responsible for the failure of granuloma to contain infection may be: first, the failure to generate efficient T cell response to *M. tuberculosis* in immunocompromised subjects and secondly, genetic factors. Studies based on racial variation in the susceptibility to TB and twin studies emphasized the imporatance of genetic factors in reactivation of TB. Fernando and Britton (2006) enumerated at least 14 genes that have been assosicated with disease reactivation and among them HLA has been studied extensively.

Both CD4+ and CD8+ T cells recognize *M. tuberculosis* peptides presented by major histocompatibility complex (MHC) class I and class II molecules. In humans, MHC is known as human leukocyte antigen (HLA). In the same report it has been emphasised that HLA has an association with susceptibility to tuberculosis in different populations. Attempts have therefore been made to identify immunodominant epitopes of *M. tuberculosis* antigens for vaccine purpose based on their recognition of HLA.

1.6.1 Immunodominanat T-cell antigens: Answer for new vaccine?

The protective T-cell response to tuberculosis is usually antigen-specific. Hence, studies have been carried out to identify immunodominant antigens for development of effective vaccines. Since T cell antigen recognition is dependent on major histocompatability

complex (MHC), the search for immunodominant antigens have been diverted to HLA binding epitopes.

1.6.1.1 Antigen 85 complex

Secretory proteins are potential targets for vaccine design as they are the first to interact with immune cells and generate the immune response. A number of culture filtrate proteins have been identified that not only contribute to pathology of the disease but can also generate an immune response. The proteins of antigen 85 complex are a group of secreted antigens which include Ag85A, Ag85B, Ag85C that code for fbpA, fbp B and fbpC respectively. In addition a fourth gene, named fbp D and encoding MPT51 who's primary structure is similar to other members of the Ag 85 complex was also identified. These are a major constituent of proteins secreted in the cutlture of mycobacteria on the synthetic, liquid Sauton medium. They are mycolyl transferase enzymes and transfer mycolyl residue from one molecule of TMM to another TMM (forming TDM or cord factor which enhances virulence) and arabinogalactan. They also have fibronectin binding property that enhances the virulence by helping in adherence and dissemination of organism in tissue (Wiker and Harboe, 1992).

1.6.1.2 Region of difference (RD region)

The studies based on the comparison of genomes revealed certain regions of the genome that are present in *M. tuberculosis* strain but were found to be absent in *M. bovis* BCG substrains and several non-tuberculosis mycobacteria (NTM), including *M. avium* (Mahairas et al., 1996; Gordon et al., 1999; Behr et al., 1999) . These gene segments are known as regions of difference (RD). The protein encoded by these RD regions are called RD proteins. Several studies have now been carried out to study the potential of these RD antigens in generating cell mediated and/or humoral immune response.

Table 1.1 Characteristics of regions of differences (RD) present in Mycobacterium tuberculosis.

RD	Size of ORF (kb)	ORF (number)	ORF (range)	Gene Segment(s)	ORF (bp range)
1	9.5	9	Rv3871-Rv3879c	160	7534–16989
2	5.6	11	Rv1978-Rv1988	88–89	14211–8598
3	9.3	14	Rv1573-Rv1586c	70	7677–16923
4	1.9	3	Rv0221-Rv0223c	12	17432–19335
5	2.8	5	Rv3117-Rv3121	135	27437–30212
6	12.8	11	Rv1506c-Rv1516c	65	23614–36437
7	~9.0	8	Rv2346c-Rv2353c	103	17622–26584
8	3.4	4	Rv0309-Rv0312	16	17018–20446
9	18.3	7	Rv3617-Rv3623	153–154	21131–2832
10	3.0	3	Rv1255c-Rv1257c	55	3689–6696
11	28.8	5	Rv3425-Rv3429	145–146	30303–1475
12	2.0	4	Rv2072c-Rv2075c	93	9301–11331
13	~11.0	16	Rv2645-Rv2660c	118	12475–23455
14	~9.1	8	Rv1766-Rv1773c	79	30573–39642
15	12.7	15	Rv1963c-Rv1977	88	1153–13873
16	7.6	6	Rv3400-Rv3405c	145	5012–12621

ORF : Open reading frame Source: Based on reference, Behr et al., 1999.

1.6.1.3 Region of Difference 1

The RD 1 region codes nine open reading frames (ORF) including Rv3871-Rv3875 of size 9.5kb (Table 1). This region is of particular importance not only because it is consistently absent in all the BCG strains but also because the deletion of the RD-1 fragment from *M. tuberculosis* causes loss of its virulence, whereas the inclusion of this region into *M. bovis* BCG or *M. microti* resulted in increased virulence and survival properties (Behr, 2002; Lewis et al., 2003) Two ORFs included in this region are Rv3874 which encode 10-kDa culture filtrate protein (CFP-10) and Rv3875 which encodes the 6-kDa early secreted antigenic target (ESAT-6) protein. Both these proteins induce T cell mediated response (Weldingh et al., 1999). Kamath et al. (2004) in their study on mice model observed that T cells were activated by CFP-10 epitopes and were recruited in large number in early stage of infection in lung which resulted in large amounts of IFN-γ.

This property of CFP-10 makes it a potential candidate for diagnostic and vaccine purpose. Both ESAT-6 and CFP-10 play crucial role in the persistence of mycobacteria in macrophages by downregulating the production of reactive oxygen species (ROS) inside the macrophages; which in turn dampens the NF-κB transactivation property.

1.6.1.4 Phospholipase C

Phospholipase C (PLC) is known to be an important virulence factor and its role in pathogenesis of organisms like *C. perfringens* (Titball, 1998; Flores-Diaz and Alape-Giron, 2003) and P. *aeruginosa* (Titball, 1998; Songer, 1997) has been established. In mycobacterium tuberculosis, phospholipase family consists of four closely related genes encoding PLCs. The plc-A, plc-B and plc-C form an operon and have been found to be relevant in virulence of *M. tuberculosis*. However the fourth gene plc-D which is located in a different region than the other three genes did not seem to have very significant role in virulence. Phospholipase C has recently been identified by potential drug target by Bakala N'goma et al. (2010). They expressed all the genes of phospholipase C family and expressed them using Mycobacterium smegmatis as expression system. These enzymes exhibited cytotoxic activity towards macrophages via direct and indirect enzymatic hydrolysis of cell membrane.

1.6.1.5 Region of Difference 12

Mustafa et al. (2011) in their study analyzed the immune response to various peptide pools of the RD region and observed three type of responses: 1) Response with Th1 bias using peptides of RD1, RD5, RD7, RD9 and RD10; 2) Response with Th2-bias in case of peptides from RD12, RD13 and RD15); and 3) Without any Th1/Th2-bias response against peptides derived from RD4, RD6 and RD11. The study emphasizes on the possible role of RD proteins with Th1 and Th2 biases in protection or pathology of the disease respectively. In another study the same group revealed that the peptide pools of RD1 region induced the production of IFN-γ whereas RD12pool and RD13 pool induced IL-10 production. The study therefore highlights the importance of antigens of RD1

region in generating a protective immune response and that of RD12 and RD13 region in inducing pathogenesis.

1.7 DEFINITION OF THE PROBLEM

In the light of what is already known in the literature, vaccine development and improved diagnostics for tuberculosis still remains a major challenge. Despite extensive research in the field of tuberculosis immunology several aspects pertaining to immune response and pathogenesis remain undeciphered. Unraveling the key aspects of the biology of the disease and protective immune response against the disease would help in designing new therapeutic approaches. The rationale of the present study was based on the following observations made from the literature that:

a) Monocytes and granuloma being important factors in disease progression and treatment, it is important to understand their role in tuberculosis. Specifically, it would be interesting to study the differences in expression of various proteins in monocytes of patients and compare them with their respective household contacts and controls.
b) Furthermore, how do the cytokines that are produced in patients, household contacts and controls influence the ability of monocytes in granuloma formation.

Based on the above rationale the objectives of the present study were the following:

1) Study of the immune response in tuberculosis patients, their corresponding household contacts and healthy controls at the basal level and following stimulation with antigen/peptide.
2) Study of *in vitro* granuloma formation from monocytes of tuberculosis patients and healthy controls
3) Study of proteomic profile of tuberculosis patients and household contacts.
4) Study of antigen/ peptide specific CTL (ELISPOT) assay of tuberculosis patients and household contacts.

CHAPTER 2

IMMUNE RESPONSE IN TUBERCULOSIS PATIENTS, HOUSEHOLD CONTACTS AND HEALTHY CONTROLS

"Go to your task with love in your heart, and you will Go to it light-hearted and cheerful".

James Allen

CHAPTER TWO

IMMUNE RESPONSE IN TUBERCULOSIS PATIENTS, HOUSEHOLD CONTACTS AND HEALTHY CONTROLS

2.1 INTRODUCTION

Following mycobacterial infection, the macrophages and dendritic cells activate the CD4+ T cells. These Th cells further differentiate into Th1 or Th2 type of cells depending on the expression of transcription factors. The Th1 (IFN-γ, IL-2, TNF-α) type of cytokines are known to control early mycobacterial infection, whereas the Th2 (IL-4, IL-6, IL-10) type of cytokines oppose the role of Th1 cytokines and aggravates the pathogenesis of the disease. Normally there is a fine balance between Th1 and Th2 type cytokines in the body. Following mycobacterial infection, the predominant Th1 type of cytokine response that occurs at an early stage can lead to the eradication of the disease (Shi et al., 2003).

The interaction of T cells with bacteria laden macrophages orchestrates the immune response to tuberculosis. The protective adaptive immune response to mycobacterial infection involves the proliferation of CD4+ and CD8+ effector T cells. IFN-γ, produced by both these cells is known to play a central role in elimination of tuberculosis. Following mycobacterial infection, the alveolar macrophages produce interleukin 12 (IL-12), which is a heterodimer of IL-12 p40 and IL-12 p35. IL-12 then stimulates T cells and Natural Killer cells (NK cells) by binding to IL-12Rβ1 in combination with IL-12Rβ2. The T cells thus activated then produce IFN-γ. IFN-γ in turn binds with high affinity, to IFN-gamma receptor 1 (IFNGR1) which then dimerizes with IFN-gamma receptor 2 (IFNGR 2) ultimately leading to the transcription of IFN-γ regulated genes (Shuai et al., 1993). Tumor Necrosis factor- alpha (TNF-α) activation is one of the results of IFN-γ activation in addition to further upregulation of IL-12 production.

IL-2, another Th1 cytokine, is a potent T cell growth factor. Apart from Th1 cells, the presence of IL-2^{+}/IFN-γ^{+} CD8+ T cells have also been shown to afford protection against

TB (Caccamo et al., 2009). T cytotoxic lymphocytes (CTL) further makes a crucial contribution to the protective immune response not only through the early burst of IFN-γ that is essential for generation of Th1 effector cells but also by killing the tubercle bacilli within cells directly through a granule dependent lysis of the bacteria within the infected macrophages (Das et al., 2001; Stenger et al., 1998).

Effective T cell proliferation and activation is facilitated by IL-2 which in turn is produced by activated T lymphocytes. On the basis of their effector function, proliferative capacity and migration into lymphoid or peripheral tissues, they may be classified into central memory T cells (T_{CM}) and effector memory cells (T_{EM}) respectively (Mueller et al., 2013). The central memory cells predominantly produce IL-2 whereas effector memory cells secrete both IFN-γ and IL-2. These memory cells are crucial in generating rapid and elevated immune response to the pathogen and hence are taken into consideration while screening the antigens/peptides for vaccine development purpose. Suter-Riniker et al., (2011) in their study on latent tuberculosis infected individuals observed that the dominance of CD4+ T cells shifted from those secreting both IL-2 and IFN-γ to those secreting only IL-2 during and following TB treatment. This type of response can be correlated with the elimination of bacteria, suggesting that the strong responders, now, may not require further prophylactic therapy.

Besides IFN-γ and IL-2, TNF-α contributes significantly to granulomatous response in tuberculosis. TNF-α is also known to upregulate the expression of adhesion molecules that would recruit the mononuclear cells to the granulomatous lesion (Jacobs et al., 2000). Studies have also implicated the role of TNF-α in apoptotic mechanisms leading to bacterial growth inhibition. It is known that TNF-α also upregulates iNOS expression in synergy with IFN-γ, which is a crucial step for containing the infection (Schon 2004).

However, excessive TNF-α can aggravate the pathology of the disease. Studies have associated extensive cavity formation with high levels of TNF-α in Bronchoalveolar lavage (BAL) fluid of active tuberculosis patients. In such cases, the pro-inflammatory effects of TNF-α lead to tissue damage and necrosis of tuberculosis lesions. TNF-α also enhances fibroblast collagenases that inhibit collagen synthesis by fibroblasts (Toossi,

2000). The tissue damaging activity of TNF-α is enhanced further by the release of reactive oxygen and nitrogen species. Hence although TNF-α is an important component of protective immunity to tuberculosis, excessive TNF- α can enhance disease pathology.

In normal conditions a fine balance between pro- and anti- inflammatory cytokines is maintained in the body. However following infection, the pro-inflammatory cytokines get elevated to eliminate the infection. Here, anti-inflammatory cytokines rescue the body from tissue damage due to inflammation. However, it is also seen that anti-inflammatory cytokines in this process might favor the survival of the pathogen. IL-10 and TGF-β are two key cytokines of anti-inflammatory response to tuberculosis. IL-10 produced by Th2 cells not only inhibits T cell proliferation but also antagonizes the production and response of IFN-γ (Bonecini-Almeida et al., 2004). TGF-β is a potent fibrogenic cytokine which acts synergistically with IL-10. Fibrosis of granuloma by TGF-β could either be beneficial in containing the infection or on the other hand, may also lead to increased cavitation and subsequent reactivation of tuberculosis (Oral et al., 2006)

2.1.1 Regulatory T cells

It is well known that T cells predominate the protective immune response to tuberculosis. Besides CD4+ T helper cells and CD8+ cytotoxic T cells, another group that has now gained a significant position in tuberculosis immunology is the Regulatory T cells (Tregs). They are known to secrete suppressive cytokines like TGF-β and IL-10 and dampen the immune response to various pathogens including *M. tuberculosis* (Garg et al, 2008).

The ability of the host to distinguish self from non-self is known as ‘self tolerance’ and a breakdown in self tolerance leads to cancer or autoimmune diseases. Hence, despite the generation of vigorous immune response to combat infections or outgrowth of tumor cells, the body’s immune system has extensive built-in defense mechanisms to prevent attack on its own healthy tissue. The elimination of the self reactive T – and B-lymphocytes during negative selection in the thymus is the first line of ‘self- tolerance’ and is called ‘central tolarence’. However, those immune cells that escape this central

tolerance eventually have to be controlled by peripheral mechanisms. The concept of existence of suppressor T cells that would render protection against the unwanted immune response was postulated long back. However, due to various inconsistencies in the findings and many unknown aspects of their fundamental biology the field was long dormant. With the identification of regulatory T cells (Tregs) the subject of suppressor T cells has again gained importance once again.

The expression profile of Tregs include CD25, CD62L and specific CD45 isoforms in addition to transcription factor Foxp3 which is essential to establish a functional regulatory T (Treg) cell lineage (Sakaguchi et al., 2008). There are two types of Tregs: 1) thymus derived $FoxP3^{+}$ Treg cells i.e. "natural Treg cells" and 2) those derived peripherally generated outside thymus "adaptive Treg cells" (Haidukov and Zurochka, 2011). Foxp3+ T regulatory cells and its role have been studied extensively in many diseases and more recently in the field of tuberculosis.

2.1.2 Immunosuppressive functions of Tregs

Although the source of IL-10 and TGF-β is not limited to Tregs, they have been known to exert their immunocuppressive effects by producing IL-10 and/or TGF-β. Another important mechanism by which Tregs exert suppressive effect is decreasing the availability of IL-2 cytokine. Tregs consititutively expresses CD25 receptor which in turn has high affinity for IL-2 produced by effector cells thereby preventing the IL-2 availability for proliferation and differentiation (Pandiyan 2007). It also similarly prevents the differentiation of CD8+ T cells into cytotoxic effector cells while sparing its IFN-γ producing ability (Mempel et al., 2006). Direct apoptotic killing of T effector cells by Tregs through the release of granzyme B and perforin have also been demonstrated (Grossman et al., 2004)

2.1.3 Role of Tregs in tuberculosis: Friend turning foe

Although Tregs have been known to be „master regulators" of immune response and prevent tissue damage, there is plenty of evidence suggesting that *M.tuberculosis* can induce regulatory T cell cytokines for its own benefit. Shafiani et al. (2010) observed that following *M. tuberculosis* infection, proliferation of Tregs did not take place until the bacilli was transported to the pulmonary lymph node (pLN) although *M. tuberculosis*-specific Tregs have high propensity to proliferate. *M. tuberculosis*- specific Tregs can delay the priming of effector CD4+ and CD8+ T cells even when they are present in very small numbers in the lymph node. In the same study it was also demonstrated that the bacterial load was increased when FoxP3+ CD4+ T cells were transferred to *M. tuberculosis* infected mice in comparison to those mice to which *M. tuberculosis*- specific FoxP3- CD4+ T cells were transferred.

Besides delaying the adaptive T cell response, the suppressive effect of Tregs on *M. tuberculosis*-specific Th response have been demonstrated in mice (Scott-Browne et al., 2007) as well as human (Singh et al., 2012) studies. Singh et al.(2012) in their study on pulmonary tuberculosis patients suggested the role of PD1 (Programmed death molecule 1) molecules preferentially expressed on Tregs in apoptosis of protective T cells that produce IFN-γ. This effect was further confirmed by the increase in IFN-γ producing T cells subsequent to blocking of PD1-PD1 ligand pathway.

An alternative way by which Tregs dampen the Th1 response is to produce anti-inflammatory cytokines like IL-10 and/or TGF-β. Both these cytokines, deactivate macrophages and modulate proinflammatory cytokines. The suppressive effect of Tregs was also observed by Sharma et al. (2009), whose study on BAL of tuberculosis patients observed that FoxP3+ T cells predominantly produced IL-10 which in turn may decrease T-cell proliferation. Garg et al. (2008) in their study also demonstrated that the *M. tuberculosis* antigen, mannose-capped lipoarabinomannan resulted in the expansion of Tregs that produced significant amount of TGF-β and IL-10. They also observed that the depletion of Tregs from PBMCs increased the frequency of *M. tuberculosis*- specific CD4+ IFN-γ+ T cells.

M.tuberculosis is infact known to facilitate Treg proliferation by inhibition of apoptosis as well as by LXA4 mediated proliferation. Inhibition of apoptosis also decreases cross-presentation of *M. tuberculosis* antigen by bystander uninfected DC, which may have more potent stimulatory activity than the infected DCs. Also LXA4 induction by *M. tuberculosis* may directly promote Treg proliferation through interaction with the aryl hydrocarbon receptor, AhR, which is a ligand activated transcription factor expressed on DCs and T cells. (Ho and Steinmann, 2008).

Studies are now being carried out to gain an in depth knowledge of the immunosuppressive role of Tregs in tuberculosis patients. It has been reported that Treg cells are expanded in tuberculosis patients as compared to healthy controls and result in correspondingly different disease states (Li et al., 2007). Though studies have been conducted to study Foxp3 positive T cells, specifically CD4+ T cells in tuberculosis patients, no study appears to have been done on CD8+ Tregs. In the present study we have analysed the Foxp3 expression in tuberculosis patients at the basal level as well as following whole antigen Ag85A and CFP-10 peptide (HLA-B*44 specific). Simultaneously we have also evaluated the mRNA levels of Th1 and Th2 type of cytokines produced by PBMCs' of active tuberculosis patients in comparison to their HHC at the basal level and following in vitro stimulation.

2.2 MATERIALS AND METHODS

2.2.1 Study population

Newly diagnosed tuberculosis patients with roentgenographic findings (chest X-ray) consistent with TB and positive sputum positive for AFB (acid fast bacilli) along with their asymptomatic healthy household contacts (HHC) were included in this study. All the TB patients (from SSG hospital, Vadodara) had a negative human immunodeficiency virus serologic test. Asymptomatic healthy controls were also included in the study. Cytokine mRNA levels of unstimulated PBMCs were analyzed in TB group (n=14), HHC (n=14) and healthy controls (n=14) whereas that of PBMCs activated with antigen and peptide were analyzed in TB group (n=14) and HHC (n=14). In addition, ELISPOT assay for IFN-γ release, was carried out using a commercially available kit (eBioscience Inc.,USA). 5 ml blood was collected in EDTA vacutainer. All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S. University of Baroda. Written informed consent was obtained from all subjects

2.2.2 Recombinant antigen and peptide

Antigen 85A

An *Escherichia coli*, recombinant clone (pVLExpMtbAg85A) expressing antigen (Ag) 85A of *M. tuberculosis* was a kind gift of Prof. V.K. Chaudhary, UDSC, New Delhi, India. The clones were grown in Luria Broth (LB) containing ampicillin (100 μg/ml). The recombinant vector (pTriEx Neo 1.1, Novagen) was used for transforming *E. coli* BL21 (DE3) cells following the protocol for transformation protocol as described by Sambrook et al., (2001). Transformants were screened on the basis of ampicillin (100 μg/ml) resistance Plasmid isolation was carried out from this clone using alkaline lysis method as described by Sambrook et al., (2001). To verify the transformation of the recombinant clones were analyzed by restriction enzyme digestion with restriction enzymes for excision of the Ag85A gene and PCR analysis. Ag 85A gene was amplified from the using specific primers.

Gene/ transcript	F:Forward, R:Reverse	Amplicon size (bp)	Annealing temp. (˚C)	Extension time	NCBI Accession number
Ag85A	F:5'GCCATGGCGTTTTCCCGGCCG GGCTTGCC 3' R:5'GATCTAGGCGCCCTGGGGCG CGGG 3'	900	66	30 sec	NC_000962.3

PCR reaction system for Ag85A amplification

Reaction Components	Volume (µl)
R.O water	17
10X Taq polymerase buffer	2.5
dNTP mix (2.5 mM each)	0.5
Forward primer (10 pmol/µl)	1.25
Reverse primer (10 pmol/µl)	1.25
Taq polymerase (1 U/µl)	0.5
cDNA	2
Total volume	25

Conditions for Ag85A specific amplification

Steps		Temp (˚C)	Time	No. of cycles
1	Pre-cycle denaturation	95	6 min	1
2	Denaturation	95	45 s	29
3	Primer annealing	66	30 s	
4	Primer extension	72	45 s	
5	Post-cycle elongation	72	6 min	1

Agarose gel electrophoresis

The PCR product was analyzed by electrophoresis in 0.8% agarose gel in 0.5X TBE followed by staining with (Ethidium Bromide) EtBr and viewing under UV.

Composition of TBE (5X): for 1 L solution

Tris 54 g

Boric acid 27.5 g

EDTA (0.5 M) 20 ml

Sequence analysis and bacterial expression of Ag 85A using IPTG induction

Amplicons obtained with the specific primers were confirmed for their identities by sequencing them. Overnight grown culture of recombinant Ag 85A clone was prepared by inoculating 5ml Luria-Bertanni Broth (LB) with ampicillin. The following day 50µl of the culture was inoculated in another 5ml LB with ampicillin and incubated for approximately 2 hours. The optical density (OD) of the cells was measured with a UV-Visible spectrophotometer (Thermo, USA). When the OD600 reached between 0.6 – 0.8, the cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma Aldrich, India) to a final concentration of 0.5mM and incubated at 37ºC with shaking at 200rpm. A volume of 1ml of the induced cultures was collected at t=0, t=3h, t=6h and t=9h post-induction. The cells were harvested from the collections by centrifugation at 10,000 rpm for 1min. The pellet was re-suspended in Laemmli Buffer (1X) for sample loading on SDS-PAGE and was analysed for protein profile accordingly.

Analysis of Ag 85A gene expression by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Cell lysate samples were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (β-mercaptoethanol, 10%).

Reagents for SDS-PAGE

Gel stock solution (30%, 100 ml)

Acrylamide	29 g
Bis acrylamide	1 g
Distilled water (DW)	100 ml

Resolving gel

DW	1.9 ml
30% gel stock	1.7 ml
1.5 M Tris-HCl (pH 8.8)	1.3 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	7.5 µl

Stacking gel

DW	1.4 ml
30% gel stock	0.33 ml
1 M Tris-HCl (pH 6.8)	0.25 ml
10% SDS	20 µl
10% APS	20 µl
TEMED	5 µl

Sample buffer (5X, 10 ml)

250 mM Tris-HCl (pH 6.8)

500 mM β-Mercaptoethanol

10% SDS

50% Glycerol

0.5% Bromophenol Blue

Tank Buffer (5X, 1 L)

Tris base	15.1 g
Glycine	94 g
SDS	10 g

SDS-PAGE gels were stained overnight with gentle shaking in approximately 200ml of a staining solution containing 45% methanol, 10% glacial acetic acid, 0.2% Coomasie Brilliant Blue. The stain was discarded and the gels were destained with approximately 600 ml destaining solution containing 45% methanol and 10% glacial acetic acid.

Purification of Ag85A using Nickle affinity chromatography

A 25ml volume of LB/amp was inoculated with 250µl of a overnight grown culture of Ag85A cloned in BL21(DE3) and incubated for approximately 2 hours at 37ºC with shaking at 200rpm. The cultures were induced with IPTG. The cell pellet harvested at 10,000 rpm for 1 min following IPTG induction was resuspended in 1X SDS- PAGE sample buffer without bromophenol blue dye and the recombinant protein was purified using Nickle affinity chromatography.

Nickel slurry (100µl) was centrifuged at 5000xg for 5 mins, supernatant was removed and again washed with 1 ml distilled water. Subsequently the slurry was resuspended in 1 ml of Equilibration Buffer I without imidazole and centrifuged at 5000xg for 5 minutes at 4ºC, the supernatant was discarded and the culture pellet was resuspended in the slurry and incubated for 2 hours at room temperature. The slurry containing Ni-complexed protein was then pulled down by centrifugation at 5000xg for 5 minutes at 4^0C and the unbound protein supernatant is removed. Equilibration buffer II was then added to the remaining mixture and was incubated on ice for 30 mins and subsequently centrifuged at 5000xg for 5 minutes at 4^0C. The pellet was washed twice using wash buffer with intermittent incubation on ice for 30 mins and centrifugation at 5000xg for 5 minutes at

4^0C. 750 µl Elution buffer I was then added to this and incubated for 30 min on ice, centrifuged at 5000g for 5 min at 4°C and supernatant was collected in another microfuge tube. This procedure was then repeated with Elution buffer II. The quantity of protein in the eluates was estimated by Bradford assay and approximately 10µg of the protein was loaded on the SDS-PAGE gel to analyse the quality of purified protein.

Precipitation of Ag85A protein by Chloroform Methanol Precipitation

To remove salts and detergent, the protein eluted with the help of nickel affinity was precipitated by chloroform methanol precipitation.

His-tag purified Ag 85A sample

↓

Add 4 vols of methanol (Vortex mix)

↓

Add 1vol of chloroform (Vortex mix)

↓

Add 3 vol of H_2O (Vortex mix)

↓13,300xg, 1min

Remove upper phase and discard leaving the inter-phase with the protein.

Add 400 ul methanol

↓

Add 4 (v/v) of methanol (Vortex mix)

↓13,300xg, 2min

Remove the supernatant and dry the pellet under stream of air

The precipitated Ag 85A was resuspended in DMSO, diluted in RPMI medium (Sigma-Aldrich, India) and filtered using low protein binding filter (Millipore, USA) of size 0.22µm. The protein was then quantified using Bradford assay.

Compositions of buffers

Equilibrium Buffer

TRIS pH8.5	50mM
NaCl	300mM
β-Mercaptoehaniol	10mM
Glycerol	10%
Imidazole	20mM

Wash Buffer

TRIS pH8.5	50mM
NaCl	1M
βME	10mM
Glycerol	10%
Imidazole	20mM

Elution buffer I

TRIS pH8.5	50mM
NaCl	100mM
βME	5mM
Glycerol	10%
Imidazole	50mM

Elution Buffer II

TRIS pH8.5	50mM
NaCl	100mM
βME	5mM
Glycerol	10%
Imidazole	200mM

2.2.3 Prediction of epitopes of CFP-10 using Propred I and CTLpred bioinformatic tools

The peptides used in this study were specific for HLA-B44 since this HLA type was observed frequently in our control and patient subjects. The epitopes were predicted on the basis of highest scoring peptides using Propred I software and confimed by CTLpred epitope prection tool for HLA-B*4403 were used in the study. Sequences of the antigens obtained from NCBI were used as input sequence for both the prediction tools. For Propred I, the peptides were predicted at threshold value of 4% with the proteosome and immunoproteosome filters kept on. The CTLpred server allows the user to predict epitopes using quantitative matrix (QM), Support Vector Machine (SVM) and Artificial Neural Network (ANN) approaches. The combine approach which includes epitopes predicted by ANN and SVM was employed to predict the epitopes. The cutoff score was 0.51 (default value) for ANN and 0.36 (default value) for SVM. (Bhasin and Raghava, 2004)

2.2.4 Isolation of PBMCs

Briefly, 10 ml of peripheral blood was collected in EDTA coated vials and was layered over equal volume of Histopaque-1077 and centrifuged at 400xg for 20 min, at room temperature. The mononuclear cell layer was carefully pipetted and washed twice with 10 ml phosphate buffered saline (PBS) followed by centrifugation at 400xg for 3 min, at room temperature. Total RNA was extracted using total RNA extraction kit (Bangalore genie).

2.2.5 RNA isolation

RNA was isolated using total RNA isolation kit (Bangalore Genei, India). The protocol followed was modified slightly (provided in parentheses, in the following paragraph, wherever modifications were made) to suit the laboratory conditions. All the glassware and plasticware were first rinsed with chloroform to destroy any RNase present. All the reagents used were prepared in Diethyl Pyrocarbonate (DEPC) treated and autoclaved water.

Cells (~$2x10^6$cells) were lysed with 0.5 ml (1 ml for $5x10^6$ to 10^7 cells in original protocol) of lysis buffer/denaturing solution (provided in the kit). Equal volume of water saturated phenol (acidic phenol) and 200 µl of chloroform were added and mixed properly by gentle inverting the tube several times. Following this, the mixture was kept on ice for 15 min. This was followed by centrifugation at 9300xg 20 min, at 4˚C. The supernatant was then taken into a fresh tube and equal volume of 100% isopropanol was added. The content was mixed properly and kept overnight (for 30 min in original protocol) at -20˚C, followed by centrifugation at 9300xg 20 min, at 4˚C. The pellet was resuspended in 300 µl of denaturing solution plus equal volume of isopropanol and mixed properly. The mixture was kept at -20˚C for half an hour and centrifuged at 9300xg 20 min, at 4˚C. Then, 600 µl of 75% ethanol was used to rinse the pellet by incubating at room temperature for 15-20 min.

Finally, the tubes were centrifuged at 9300xg 20 min, at 4˚C. The pellet thus obtained was dried, followed by resuspension in 100 µl DEPC treated autoclaved water.

2.2.6 cDNA synthesis

Integrity of the RNA samples was confirmed by visualizing the 28S and 18S rRNA bands following separation by electrophoresis on a 0.8% agarose gel (Fig.2.5). Following confirmation, 2 µg of the RNA was taken for subsequent cDNA synthesis.

In a tube pretreated with chloroform and subsequently autoclaved, 2 µg of RNA was taken and the volume made up to 10.5 µl with DEPC treated water, followed by addition of oligo $dT_{(18mer)}$ primer (1 µl). The contents were mixed properly and incubated at 65˚C for 10 min, followed by 2 min at room temperature. The following reagents were then added sequentially: 1 µl each of 10 U/µl RNAsin and 100 mM dithiotreitol (DTT), 4 µl of 5X reverse transcriptase buffer, 2 µl of 30 mM dNTP mix (7.5 mM each dNTP) and 0.5 µl of 100 U/µl M-MuLV Reverse transcriptase and the entire mixture further incubated at 37˚C for 1 h followed by 95˚C for 5 min. At the end of the incubation, the tubes were immediately cooled on ice for 15 min and stored at -20˚C until use.

Prior to performing PCR with the specific primers for the selected cytokines, PCR with primers specific for β-actin was performed for all the cDNA preparations. This was done in

order to confirm the integrity of the RNA preparations and also the quality of the cDNA synthesized by reverse transcription.

PCR reaction system for β-actin specific amplification

Reaction Components	Volume (µl)
R.O water	17
10X Taq polymerase buffer	2.5
dNTP mix (2.5 mM each)	0.5
Forward primer (10 pmol/µl)	1.25
Reverse primer (10 pmol/µl)	1.25
Taq polymerase (1 U/µl)	0.5
cDNA	2
Total volume	25

Conditions for β-actin specific amplification

Steps		Temp (˚C)	Time	No. of cycles
1	Pre-cycle denaturation	95	6 min	1
2	Denaturation	95	45 s	29
3	Primer annealing	66	30 s	
4	Primer extension	72	45 s	
5	Post-cycle elongation	72	6 min	1

Table 2.1. Primer sequences used for specific amplification of cytokines and FoxP3 and the corresponding amplicon sizes, annealing temperatures and extension times.

Gene/ transcript	F:Forward, R:Reverse	Amplicon size (bp)	Annealing temp (˚C)	Extention time	NCBI Accession number
β-actin	F: 5'AGCGGGAAATCGTGCGTGACA3' R: 5'CGCAACTAAGTCATAGTCCGC3'	536 bp	66	1 min.	XM_003259772
IFN-γ	F: 5'ATATCTTGGCTTTTCAGCTC3' R: 5'CTCCTTTTTACTTCA3'	256 bp	54	30 sec.	NM_000619.2
TNF-α	F: 5'CAGAGG GAA GAG TTCCCCAG3' R:5'CCT TGGTCTGGTAGGAGACG-3'	324 bp	62	30 sec.	NM_000594.3
TGF-β	F: 5'CTCCGAGAAGCGGTACCTGAAC3' R: 5'CACTTGCAGTGTGTTATCCCT3'	288 bp	60	30 sec.	NM_000660.4
IL-10	F: 5'GAAGGCATGCACAGCTCG3' R: 5'CTGCCTAACATGCTTCGAGA3'	128bp	62	30 sec.	NM_000572.2
FoxP3	F: 5'GCCCTTGGACAAGGACCCGATG3' R: 5'CATTTGCCAGCAGTGGGTAGGA3'	607bp	60	1 min	NM_014009.3

2.2.7 Reverse transcriptase PCR (RT-PCR)

The semiquantitative reverse transcriptase PCR to measure mRNA levels of cytokines was performed as described previously (Gaudana et al., 2010).The quality of the RNA samples was assessed by inspecting the 28S and 18S bands following agarose gel electrophoresis. A quantity of 2µg of each RNA sample was used with Oligo (dT18) for cDNA synthesis in a 20µl system using an M-MuLV RT-PCR kit (Bangalore Genei) following the manufacturer‟s instructions. Briefly, the RNA and Oligo (dT18) mixture was incubated at 65°C for 10 min, centrifuged briefly and kept at room temperature for 2 min. Into this RNAsin, dithiothreitol, RT buffer, deoxynucleotide triphosphate (dNTP) and M-MuLV RT were added, as instructed by the manufacturer and incubated further at 37°C for 1 h, followed by 5 min incubation at 95°C. Each of the cDNA preparations was then amplified for thirty cycles in a thermal cycler (Eppendorf, Hamburg, Germany) with

β-actin-specific primers by taking 1 µl of the cDNA in a 12·5µl system. This was used as a control for the synthesis of cDNA. Control for checking genomic DNA contamination included amplification of the total RNA without reverse transcription which did not give any amplicon (results not shown).

2.2.8 Cytokine mRNA analysis of unstimulated PBMCs

PCR amplifications of cDNA obtained from active TB patients, HHC and controls were then performed with specific primers for cytokines in a 12·5µl system with 1 µl of the first-strand cDNA, 0·2Mm deoxynucleotide triphosphates (dNTPs), 1·5mM-MgCl2 and 1·25 pmol each of the forward and reverse primers. This was run for thirty cycles after the addition 0·5U of Taq polymerase. Amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by thirty cycles of denaturation at 94°C for 45 s, annealing for 30 s at 66°C for β-actin, 54°C for IFN-γ, 62°C for TNF-α and IL-10 and 60°C for TGF-β followed by extension at 72°C for 1 min and a final extension at 72°C for 6 min. PCR products separated on a 2% agarose gel were stained with ethidium bromide (0·5mg/ml), following which densitometric analysis was carried out using AlphaEaseFC 4.0 software (Alpha Innotech, San Leandro, CA, USA) and the results are expressed as integrated density value divided by the selected area of band on the gel.

2.2.9 Cytokine mRNA analysis of stimulated PBMCs

PBMC were suspended in RPMI 1640 (Sigma-Aldrich) complete medium. An aliquot containing 2 X 10^5 cells/well was cultured in 96 well flat bottom plate with phytoheamagglutinin (PHA) (5 µg/mL), CFP-10 peptide (TAAQAAVVR) (5 µg/ mL) and Ag85A (5 µg/mL) individually. One well that was without antigen or peptide was taken as control. After 3 days, the cells were used for semi-quantitative PCR as mentioned earlier.

2.2.10 ELISPOT assay

ELISPOT assay was performed according to manufacturer's instructions using a kit (eBioscience, USA). Briefly, 96 well PVDF membrane ELISPOT plates were coated with

capture antibody and incubated overnight at 4°C. A total of 2X10^5 PBMC‟s were added per well. Ag85A and CFP-10 peptide were added at a final concentration of 5µg/ mL Negative controls consisted of wells containing PBMCs incubated with medium alone.

Positive controls consisted of wells containing PBMCs and 5 µg/mL. Plates were incubated overnight at 37°C in a 5% CO2 incubator. Next morning the plates were washed and biotinylated anti–IFN-γ detection antibody was added for 2 hours at room temperature. The plates were then washed and Avidin-horseradish peroxidase was added for 45 minutes followed by a washing with PBS-Tween (0.5%). Peroxidase substrate which was prepared by dissolving 20 mg 3-amino-9-ethylcarbazol (Sigma) in 2 mL dimethylformamide (Sigma) was diluted 1:30 in 10 mL of 0.1M sodium acetate buffer at pH 5.0. The substrate solution was then filtered through a 0.22 filter and 10 µL 30%

H2O2 was added to it immediately before use. 100 µL of substrate was added per well and the spot development was observed at room temperature. The reaction was stopped by discarding the substrate and washing the plates with PBS-Tween (0.5%). The spots were air dried and counted using a stereomicroscope.

ELISPOT ASSAY

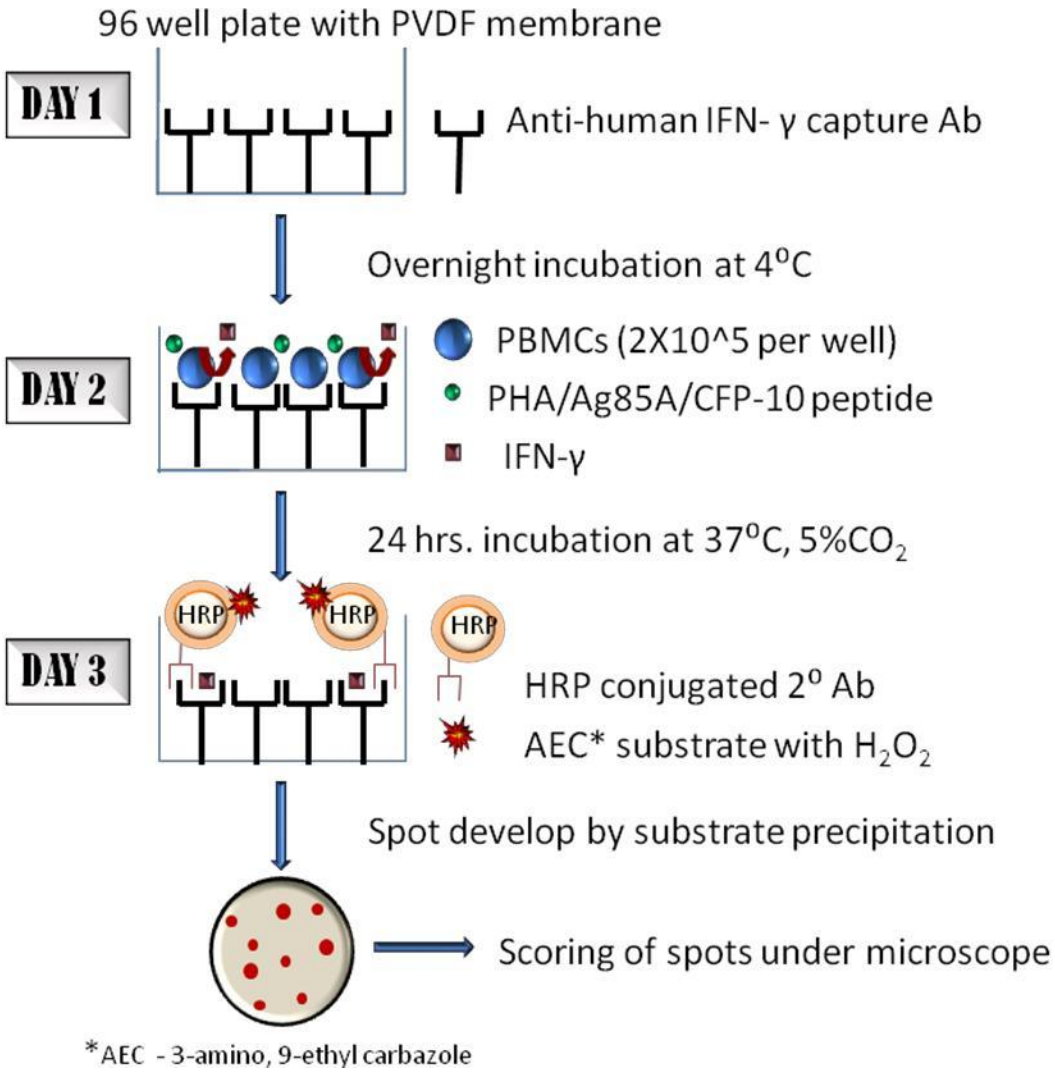


Figure 2.1 Flow chart showing ELISPOT assay

2.3 RESULTS

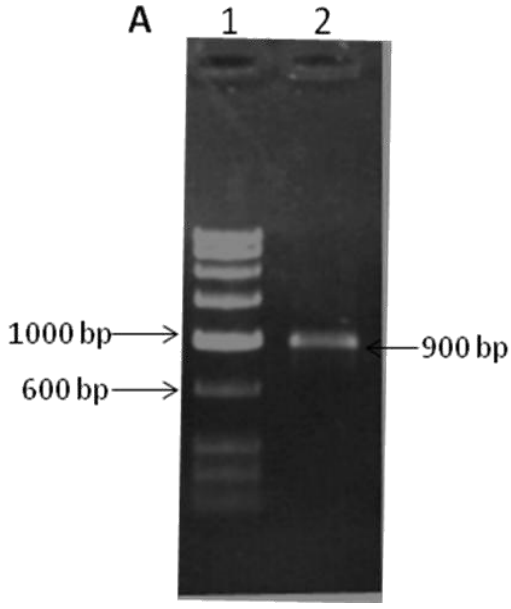
2.3.1 Cloning, expression and purification of Ag85A

Cloning of Ag85A in pTriEx 1.1 Neo expression vector

Ag85A is one of the major secreted antigens of *M. tuberculosis* that has been used extensively in studying immune response to tuberculosis. It has also been used in the diagnosis of tuberculosis. Therefore Ag85A was included in the present study to analyse the immune response of tuberculosis patients in comparison to household contacts and healthy controls. The antigen was cloned and expressed in pTriEx 1.1 Neo vector. The gene for the same was amplified from the recombinant clone pVLExpMtbAg85A. The amplicon so obtained contained the gene for Ag85A along with the coding sequence of His tag downstream of it. As can be seen from Fig.2.2A an amplicon of 900 bp was obtained from the clone which was further cloned in pTriEx 1.1 Neo expression vector (~6.6 Kbp). Fig.2.2B shows confirmation of the pTriEx1.1Neo-Ag85B by double digestion with BamH1 and Nco1 where an insert release of 900bps can be seen.

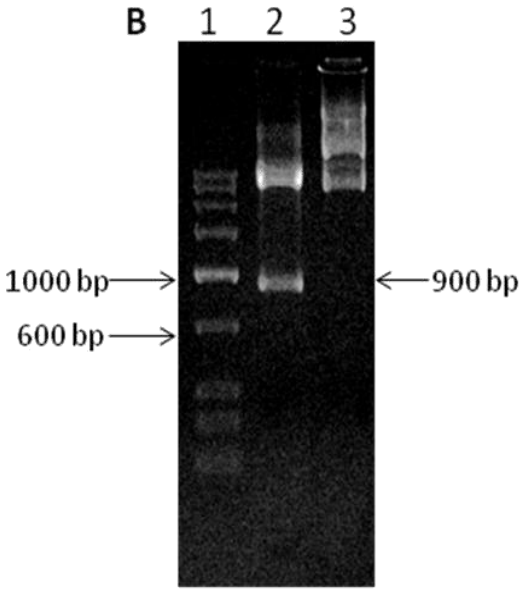
Expression of recombinant Ag 85A

The expression of Ag85A was standardized by exposure of the culture to 0.5mM of IPTG for various time period such as 3 hours, 6 hours and 9 hours. The expression of the protein was confirmed by SDS-PAGE analysis followed by silver staining. As seen in Fig. 2.3 the uninduced and induced cultures of the recombinant Ag85A clone was obtained from various time period and loaded on the SDS-PAGE gel to analyse the expression of Ag85A. The cell extract containing IPTG-induced Ag85A was resolved by 10% SDS-PAGE and stained by Coomassie. The uninduced cell-extract was used as control. The expected band of 30kDa corresponding to Ag85A was visualized. The induced protein showed maximum expression at 6 hours and therefore all cultures were thereby induced and then harvested following 6 hours of induction. Since the Ag85A protein is His tagged, Nickle affinity chromatography was used to purify the proteins.



Lane 1: Low range DNA ruler (3Kb- 300bp)

Lane 2: Amplicon of Ag85A

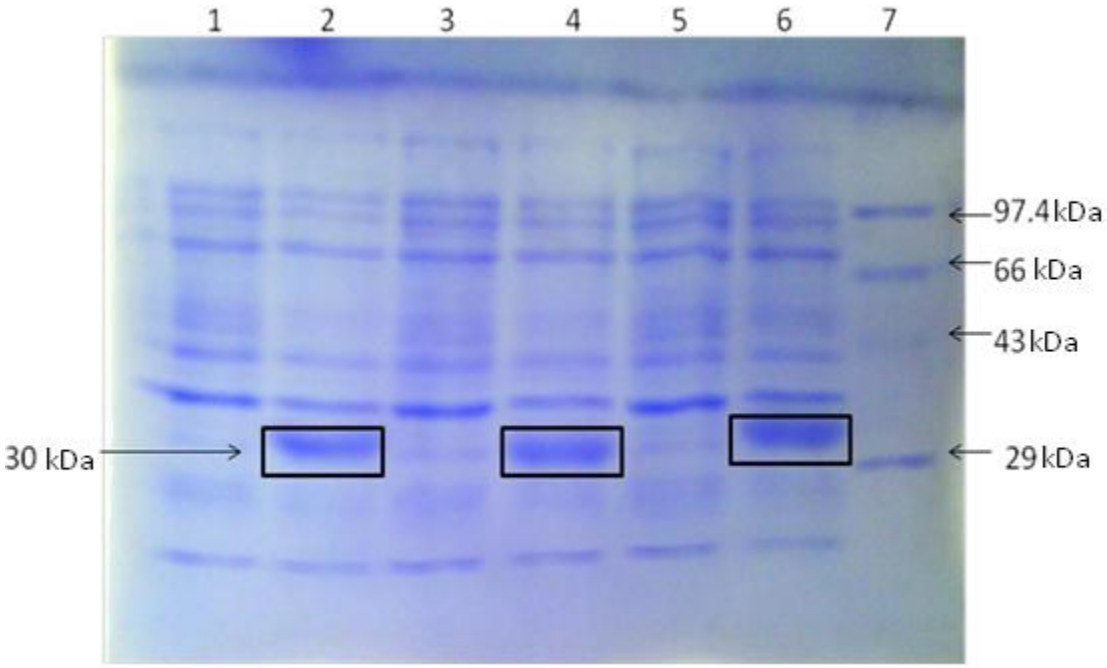


Lane 1: Low range DNA ruler (3Kb- 300bp)

Lane 2: Double digest of Ag85A cloned in pTriEx 1.1 Neo

Lane 3: Undigested plasmid of Ag85A-pTriEx 1.1 Neo

Figure 2.2 Analysis of plasmid from recombinant clone of Ag85A in pTriEx 1.1 Neo vector. Ethidium bromide stained 0.8% agarose gel electrophoresis of **A)** PCR amplified Ag85A gene from recombinant plasmid. **B)** BamH1 and Nco1 digested recombinant plasmid showing an insert release of 900bp.



Lane 1: Uninduced culture of Ag85A-pTriEx1.1 harvested at 3 hrs
Lane 2: Induced culture of Ag85A-pTriEx1.1 harvested at 3 hrs
Lane 3: Uninduced culture of Ag85A-pTriEx1.1 harvested at 6 hrs
Lane 4: Induced culture of Ag85A-pTriEx1.1 harvested at 6 hrs
Lane 5: Uninduced culture of Ag85A-pTriEx1.1 harvested at 9 hrs
Lane 6: Induced culture of Ag85A-pTriEx1.1 harvested at 9 hrs
Lane 7: Low range molecular weight marker

Figure 2.3 Commassie blue stained 10% SDS-PAGE analysis of 0.5mM IPTG induced Ag85A-pTriEx-1.1 neo clone harvested at 3hrs, 6hrs and 9hrs interval.

Purification of recombinant Ag85A using Ni Affinity chromatography

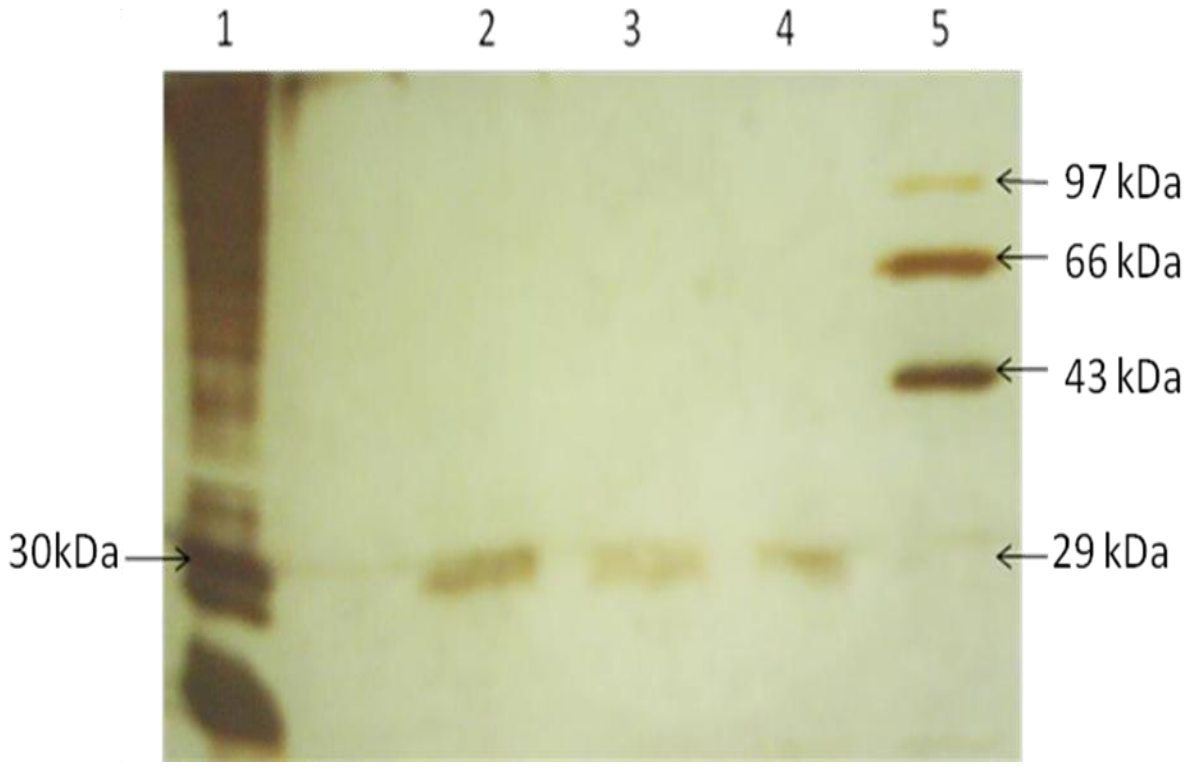
The His tagged Ag85A culture was incubated with Ni slurry, washed and then eluted with imidazole containing elution buffer. The eluted protein was analyzed on a SDS-PAGE gel. The protein was then precipitated using methanol-chloroform method and then dissolved in DMSO. The protein was then diluted in RPMI and filtered using low protein binding filter of (0.22μm).This protein preparation was quantified using Bradford assay and was used at a concentration of (5μg/ml) such that the final concentration of DMSO did not exceed 0.1% (Fig.2.4).

2.3.2 Peptide prediction of CFP-10 peptide

CFP-10 is an antigen belonging to RD1 region and has been used in several studies that demonstrate its importance for diagnosis of tuberculosis. We used bioinformatic tool, Propred-I to predict the candidate epitopes of CFP-10 for HLA-B*4403 which was found frequently in both patient and control population (Data shown in Chapter 5). The peptide giving high score with Propred-I was selected only after further confirmation using another bioinformatic tool CTL-pred.

Table 2.2 Details of CFP-10 peptide predicted by bioinformatic tool CTLpred

ORF	Sequence	Start position	Propred Score	CTLpred ANN/SVM Score
CFP-10 (Rv3874)	TAAQAAVVR	49	6	0.36/0.40



Lane 1: Induced culture of Ag85A-pTriEx1.1
Lane 2,3,4 : Ag 85A protein purified through Ni affinity chromatography
Lane 5: Low range molecular weight marker

Figure 2.4 Silver stained 10% SDS-PAGE analysis showing Nickle affinity purified Ag 85A protein from different preparations.

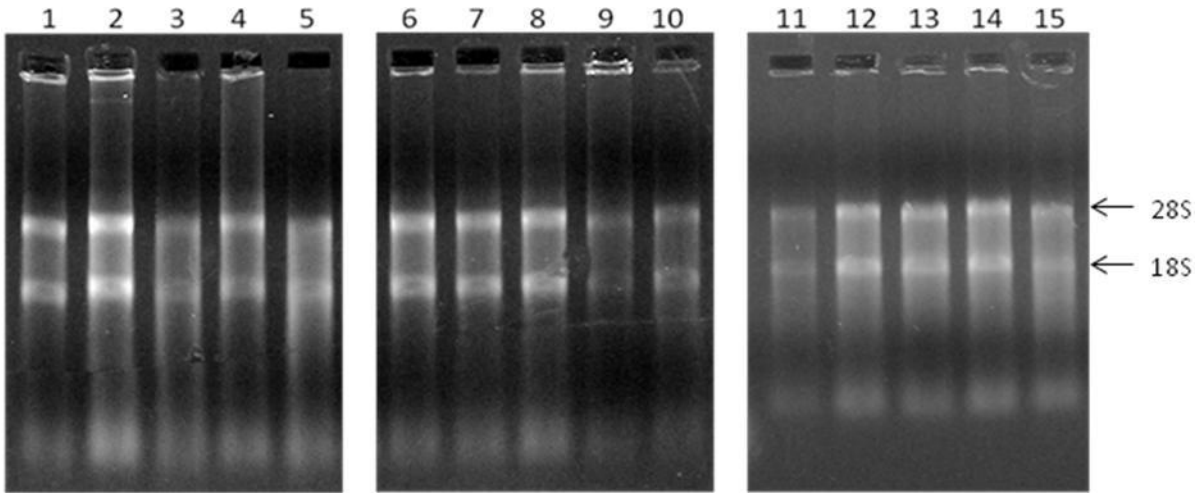
2.3.3 Analysis of FoxP3 and cytokine levels in unstimulated PBMCs

Total RNA was extracted from PBMCs obtained from active tuberculosis patients, household contacts and controls. The RNA samples were run on 0.8% agarose gel to check the integrity of DNA by visualizing 28S and 18S rRNA. The RNA was then quantified using A° 260/ A° 280 ratio and subsequently 2µg of RNA was used for cDNA synthesis. The quality of cDNA synthesized by reverse transcription was confirmed by performing PCR with β actin specific primers (Fig.2.5).

2.3.3.1 Cytokine and FoxP3 mRNA analysis

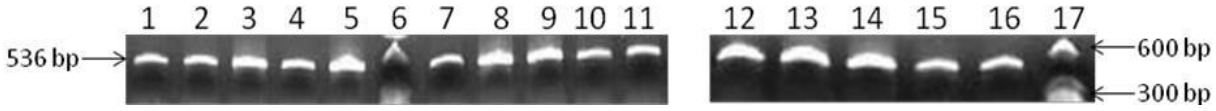
Cytokines IFN-γ, TNF-α, TGF-β, IL-10 and FoxP3 specific primers were used to investigate the mRNA levels of these cytokines at the basal level in patients, contacts and controls. The amplicons were separated on 0.8% agarose gel stained with ethidium bromide and densitometric analysis was performed using AlphaEaseFC 4.0 software. The values of the densitometric analysis were expressed as average of integrated density value per unit area (Fig.2.6).

A



Lane 1-5 : Patient samples
Lane 6-10: Household contact samples
Lane 11-15: Healthy control sample

B



Lane 1-5 : Patient
Lane 6 : Low rang DNA ruler (3Kb-300bp)
Lane 7-10: Household contact
Lane 12-16: Control
Lane 17 : Low rang DNA ruler (3Kb-300bp)

Figure 2.5 Analysis of RNA and cDNA preparation for cytokine levels. Ethidium bromide stained 0.8% agarose gel electrophoresis of A) few representative total RNA isolated from blood samples of patients, HHC and controls respectively. B) representative β- actin amplicons obtained from cDNA of patients, HHC and controls respectively.

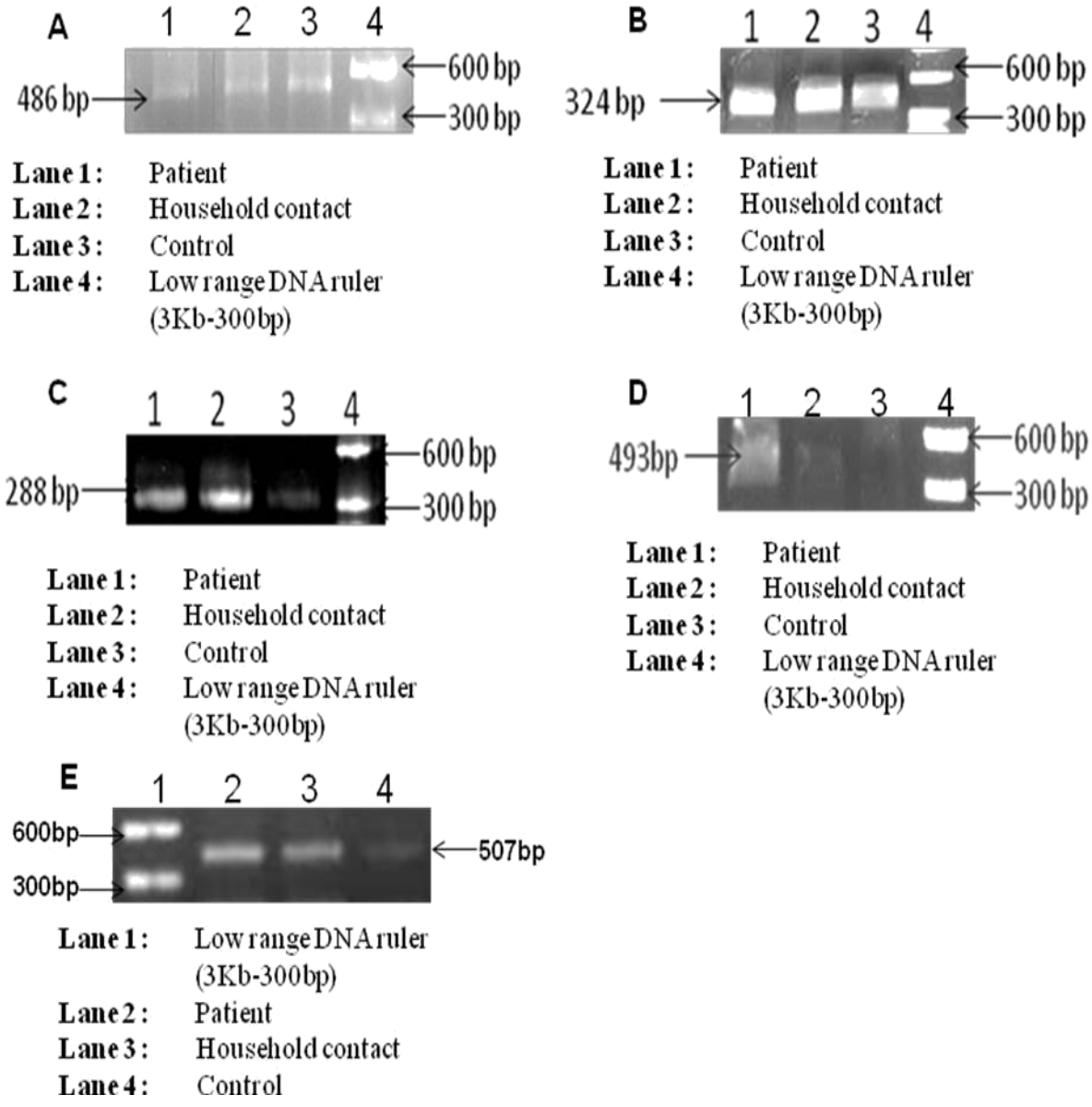
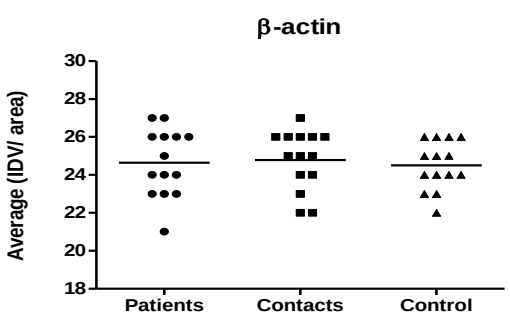
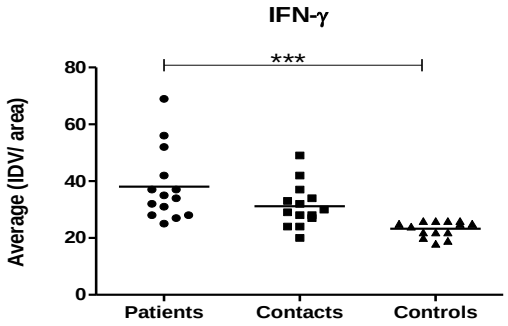
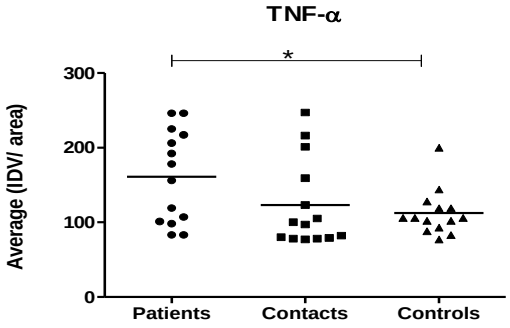


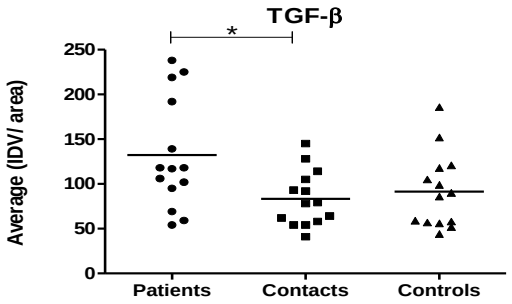
Figure 2.6 Analysis of cytokine specific amplicons of unstimulated PBMCs'.

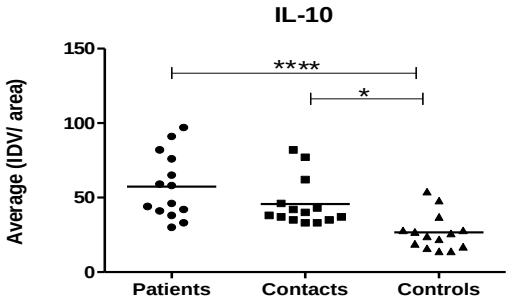
Ethidium bromide stained 0.8% agarose gel electrophoresis of semi-quantitative PCR amplicons of cytokines A) IFN-γ, B) TNF-α, C) TGF-β, D) IL-10 and E) FoxP3 using cDNA obtained from PBMC of patients, HHC and controls respectively. These gels were then sused for densitometric analysis using AlphaEaseFC 4.0 sofware.

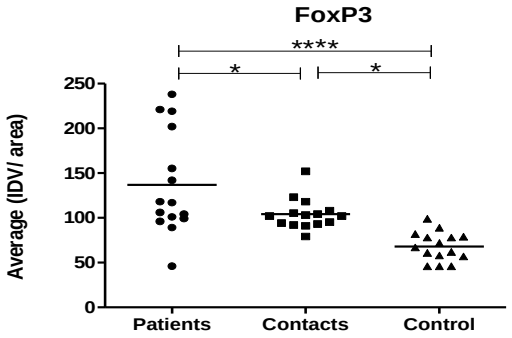

β-actin
Average (IDV/ area)
30
28
26
24
22
20
18
Patients
Contacts
Control


IFN-γ
***
Average (IDV/ area)
80
60
40
20
0
Patients
Contacts
Controls


TNF-α
*
Average (IDV/ area)
300
200
100
0
Patients
Contacts
Controls


TGF-β
*
Average (IDV/ area)
250
200
150
100
50
0
Patients
Contacts
Controls


IL-10
****
*
Average (IDV/ area)
150
100
50
0
Patients
Contacts
Controls


FoxP3
****
*
*
Average (IDV/ area)
250
200
150
100
50
0
Patients
Contacts
Control

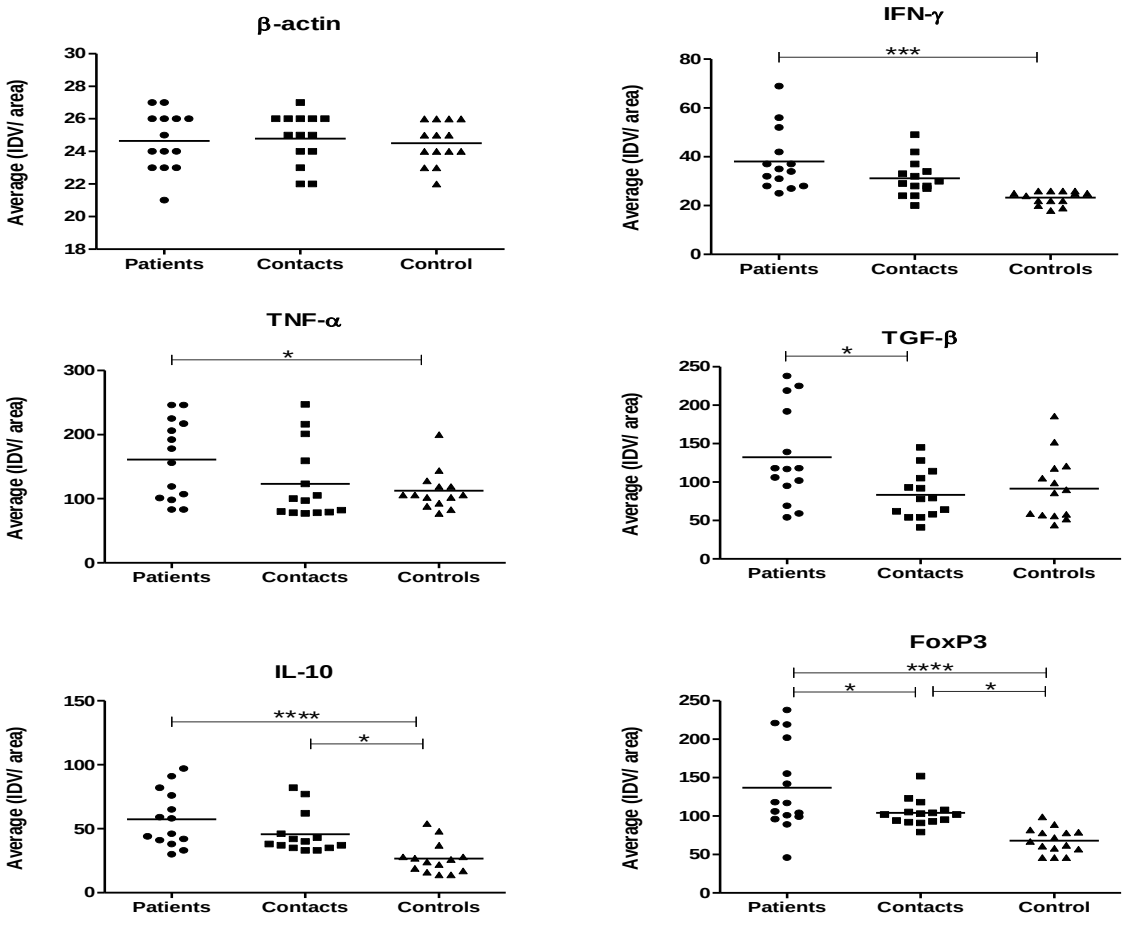


Figure 2.7 Cytokine mRNA levels of unstimulated PBMCs by semi quantitative PCR. Graph showing mRNA levels of Patients - (n=14) Contacts - (n= 14) and Controls - (n=14) for A) β-actin B) IFN-γ C) TNF-α D) IL-10 E) TGF-β and F) FoxP3. The densitometric analyses of the various cytokine specific amplicons were carried out using AlphaEaseFC software. The integrated density values (IDV) per unit area was obtained and comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

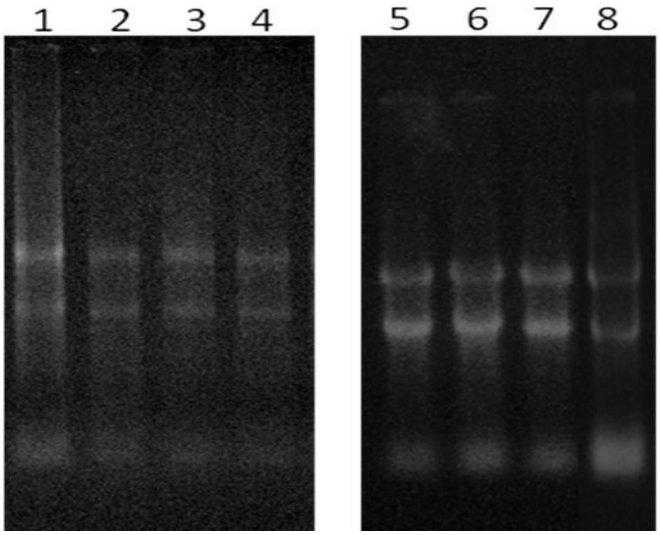
2.3.4 FoxP3 and cytokine mRNA analysis of stimulated PBMCs'

In another experiment, the PBMCs' of tuberculosis patients and controls were stimulated with mitogen PHA, Ag85A and CFP-10 peptide respectively. The unstimulated PBMC were taken as control. The PBMCs' were harvested following 24hrs of stimulation and total RNA was extracted, analysed on 0.8% agarose gel and cDNA was prepared. The cDNA integrity was determined by amplification of housekeeping β actin (Fig.2.8).

2.3.4.1 Cytokine and FoxP3 mRNA analysis

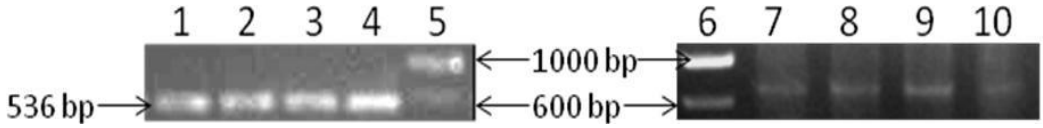
Cytokines TNF-α (Fig.2.9A), TGF-β (Fig.2.9B), IL-10 (Fig.2.10A) and FoxP3 (Fig.2.10B) specific primers were used to analyse and compare the mRNA levels of these cytokines in the PHA/ Ag85A/ CFP-10 peptide in comparision to unstimulated samples in the in patients and controls respectively. The amplicons were separated on 0.8% agarose gel stained with ethidium bromide and densitometric analysis was performed using AlphaEaseFC 4.0 software. The values of the densitometric analysis were expressed as average of integrated density value per unit area.

A



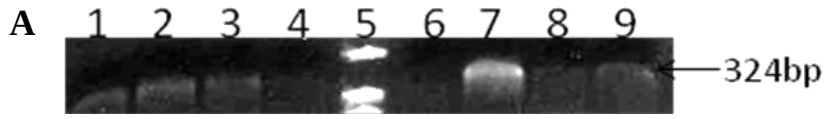
Lane 1: Patient unstimulated PBMCs'
Lane 2: Patient PBMCs' + PHA
Lane3: Patient PBMCs' + Ag 85A
Lane 4: Patient PBMCs' + CFP-10 peptide
Lane 5: Control unstimulated PBMCs'
Lane 6: Control PBMCs' + PHA
Lane 7: Control PBMCs' + Ag85A
Lane8: Control PBMCs' + CFP-10 peptide

B

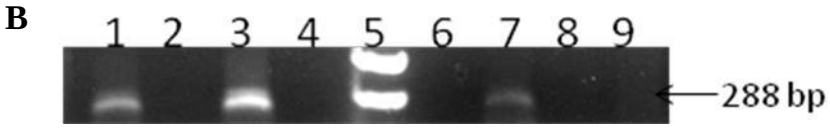


Lane 1: Patient unstimulated PBMCs'
Lane 2: Patient PBMCs' + PHA
Lane3: Patient PBMCs' + Ag 85A
Lane 4: Patient PBMCs' + CFP-10 pep
Lane 5 &6 : Low range DNA ruler **(3kd –** 300bp)
Lane 7: Control unstimulated PBMCs'
Lane 8: Control PBMCs' + PHA
Lane 9: Control PBMCs' + Ag85A
Lane10: Control PBMCs' + CFP-10 pep

Figure 2.8 Analysis of RNA and cDNA of stimulated PBMCs'. Ethidium bromide stained 0.8% agarose gel electrophoresis of A) Representative total RNA isolated from unstimulated and PHA/ Ag85A/ CFP-10 peptide stimulated PBMC of patients and controls respectively B) Representative β- actin amplicons obtained from cDNA of unstimulated and stimulated PBMC of patients and controls respectively



Lane 1: Patient unstimulated PBMCs'
Lane 2: Patient PBMCs' + PHA
Lane3: Patient PBMCs' + Ag 85A
Lane 4: Patient PBMCs' + CFP-10 pep
Lane 5: Low range DNA ruler (3kd – 300bp)
Lane 6: Control unstimulated PBMCs'
Lane 7: Control PBMCs' + PHA
Lane 8: Control PBMCs' + Ag85A
Lane9 : Control PBMCs' + CFP-10 pep

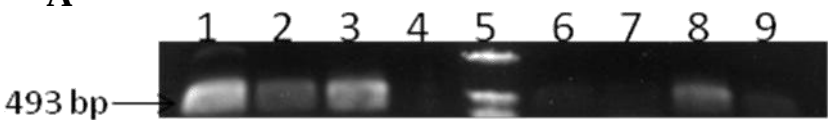


Lane 1: Patient unstimulated PBMCs'
Lane 2: Patient PBMCs' + PHA
Lane3: Patient PBMCs' + Ag 85A
Lane 4: Patient PBMCs'+ CFP-10 pep
Lane 5: Low range DNA ruler (3kd – 300bp)
Lane 6: Control unstimulated PBMCs'
Lane 7: Control PBMCs' + PHA
Lane 8: Control PBMCs' + Ag85A
Lane9 : Control PBMCs' + CFP-10 pep

Figure 2.9 Analysis of TNF-α and TGF-β specific amplicons of stimulated PBMCs'.

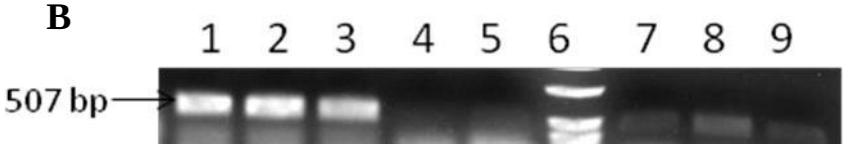
Ethidium bromide stained 0.8% agarose gel electrophoresis of semi-quantitative PCR amplicons of cytokines A) TNF-α and B) TGF-β using cDNA obtained from unstimulated and PHA/ Ag85A/ CFP-10 stimulated PBMC of patients, HHC and controls respectively. These gels were then used for densitometric analysis using AlphaEaseFC 4.0 software.

A



Lane 1: Patient unstimulated PBMCs'
Lane 2: Patient PBMCs' + PHA
Lane3: Patient PBMCs' + Ag 85A
Lane 4: Patient PBMCs' + CFP-10 pep
Lane 5: Low range DNA ruler (3kd – 300bp)
Lane 6: Control unstimulated PBMCs'
Lane 7: Control PBMCs' + PHA
Lane 8: Control PBMCs' + Ag85A
Lane9 : Control PBMCs' + CFP-10 pep

B



Lane 1: Patient unstimulated PBMCs'
Lane 2: Patient PBMCs' + PHA
Lane3: Patient PBMCs' + Ag 85A
Lane 4: Patient PBMCs' + CFP-10 pep
Lane 5: Control unstimulated PBMCs'
Lane 6: Low range DNA ruler (3kd – 300bp)
Lane 7: Control PBMCs' + PHA
Lane 8: Control PBMCs' + Ag85A
Lane9 : Control PBMCs' + CFP-10 pep

Figure 2.10 Analysis of IL-10 and Foxp3 specific amplicons of stimulated PBMCs'.

Ethidium bromide stained 0.8% agarose gel electrophoresis of semi-quantitative PCR amplicons of cytokines A) IL-10 and B) FoxP3 using cDNA obtained from unstimulated and PHA/ Ag85A/ CFP-10 stimulated PBMC of patients, HHC and controls respectively. These gels were then used for densitometric analysis using AlphaEaseFC 4.0 software.

2.3.4.2 Increased FoxP3, IL-10 and TGF-β in patients following stimulation of PBMC's

To further investigate the difference in cytokine profiles of patients and contacts we studied the mRNA expression levels following stimulation of PBMCs with whole antigen Ag85A and CFP-10 peptide. As shown in Figure 2.11, the mRNA levels for TNF-α did not increase following stimulation with Ag85A or CFP-10 peptide in either patients (Ag85A: p= 0.8918; CFP-10: p=0.91) or HHC (Ag85A: p= 0.7092; CFP-10: p=0.8583). However, both antigen and peptide specific FoxP3 levels were found to increase in patients (Ag85A: p<0.0001; CFP-10: p=0.0016) but not in HHC (Ag85A: p= 0.2071; CFP-10: p=0.0949). TGF-β on the other hand, was increased only in patients following Ag85A (p=0.0026) but not CFP-10 peptide (p=0.1671) stimulation. Conversely, TGF-β mRNA levels did not change when PBMC's of HHC were stimulated (Ag85A: p= 0.9768; CFP-10: p=0.832). IL-10 mRNA levels were increased significantly in patients following stimulation (Ag85A:p= 0.0077, CFP-10: p= 0.0091) but not in case of contacts (Ag85A: p> 0.9999, CFP-10: p=0.9966) (Fig.2.11).

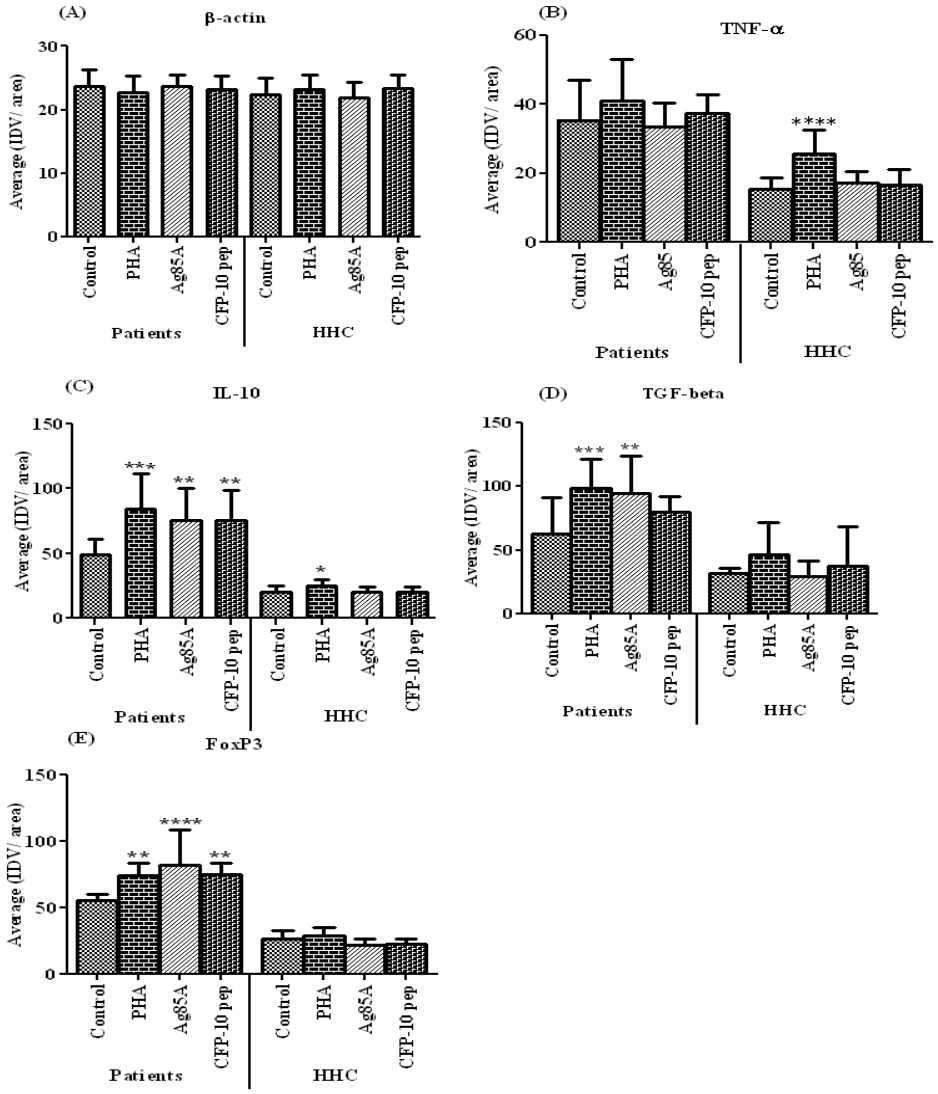


Figure 2.11 Cytokine mRNA levels of PBMCs by semi quantitative PCR following stimulation by PHA, CFP-10 peptide and Ag 85A. Graph showing mRNA levels of patients (n=14) and HHC (n= 14) for A) β-actin B) TNF-α C) IL-10 D) TGF-β and E) FoxP3. The integrated density values (IDV) per unit area was obtained and comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$.

2.3.5 IFN- γ ELISpot assay

IFN-γ production in response to CFP-10 peptide and Ag85A has been evaluated using ELISpot. Highest number of SFUs following stimulation with both Ag85A as well as CFP-10 was observed in patients followed by contacts and control (Figure 2.12). The SFUs were found to be significantly higher in patients compared to contacts (Ag85A: $p < 0.0001$; CFP-10: $p = 0.0003$) and control (Ag85A: $p < 0.0001$; CFP-10: $p < 0.0001$). The contacts in turn, had increased numbers of SFUs with respect to controls in case of CFP-10 ($p = 0.0174$). However not much difference was found in the number of SFUs between contacts and controls when stimulated with Ag85A ($p = 0.479$).

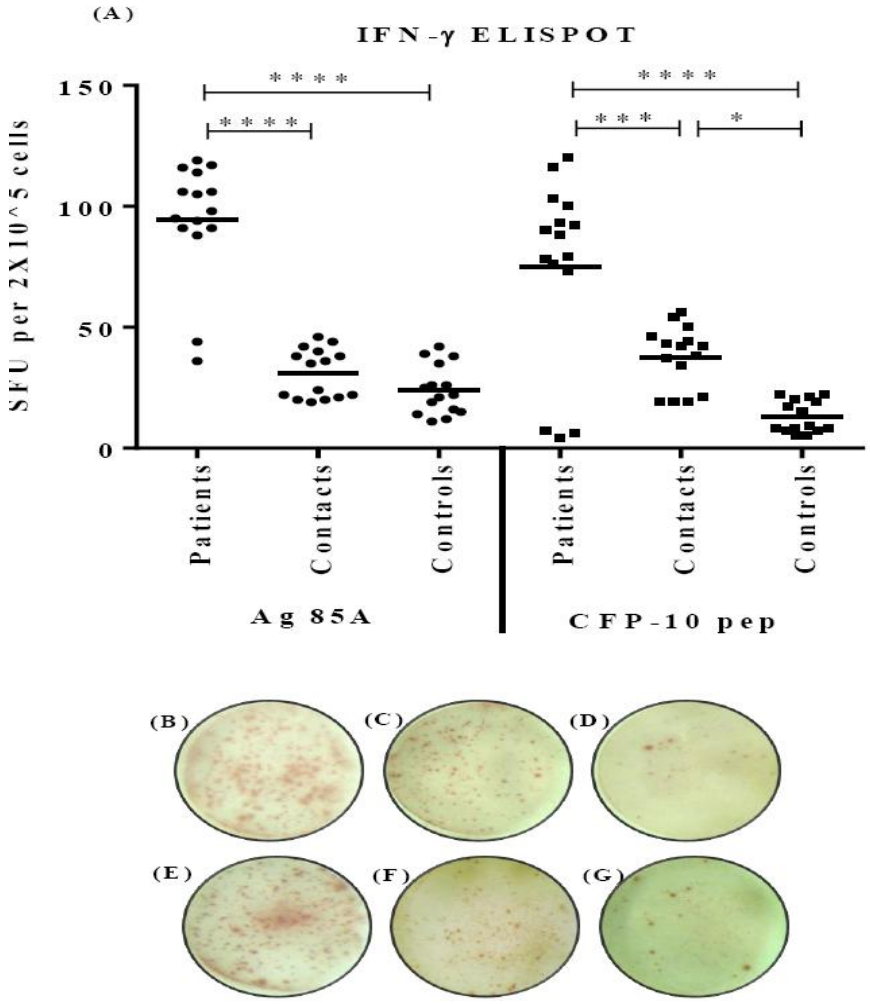


Figure 2.12 ELISPOT assay. A) Graph showing number of SFUs obtained following stimulation of PBMCs in IFNγ-ELISPOT assay. The number of SFUs' more than 5 are considered positive i.e above the dotted line. Figure shows representative pics of ELISPOT assay following Ag85A stimulation of B) Patient C) Contacts and D) Controls and following CFP-10 peptide stimulation of E) Patient F) Contacts and G) Controls. comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

2.4 DISCUSSION

T lymphocytes govern the immune response to tuberculosis. The crucial role of Th1 and CD8+ in protective immunity against the disease has already been demonstrated (Das et al., 2001; Stenger et al., 1998). Since many years defective Th1 cytokine (IFN-γ) or γ – interferon receptor has been suggested as one of the factors contributing to tuberculosis susceptibility. Vilcek et al. (1986) observed that nine out of fourteen patients with acute pulmonary tuberculosis did not elevate IFN-γ levels even following stimulation of peripheral blood cells with PHA (mitogen). Furthermore, stimulation of peripheral blood cells with PHA in the presence of exogenous IL-2 also failed to produce normal IFN-γ levels in patients with severe disease. Recently, Sundaram et al. (2013) in their case presentation study investigated a 5 year old for recurrent TB osteomyelitis of iliac bone and observed partial defect of γ – interferon receptor. The term mendelian susceptibility to mycobacterial disease (MSMD) was given to individuals such suffer primary immunodeficiency syndromedue to mutations in any of the five genes in the IL-12/ IFN-γ axis. Such individuals are susceptible to even weakly virulent mycobacteria indicating the importance of this pathway in immune response. Mutations in this case can vary from IFNGR1/IFNGR2,STAT1, IL-12 and IL-12Rβ (Döffinger et al., 2002). Sudies have correlated these mutation with susceptibility to infection. Jouanguy et al. (1999) reported small deletions in IFNGR1 rendered the individuals susceptible to infections caused by poorly virulent mycobacteria.

In the present study we therefore analysed the levels of tuberculosis patients with respect to their respective household contacts and controls to know if these patients are compromised in their ability to produce IFN-γ. We evaluated the levels of IFN-γ in PBMC of tuberculosis patients by analyzing the basal mRNA level (Fig.2.6) as well as release of IFN-γ following *M. tuberculosis* specific stimulation by ELISPOT (Fig.2.12), to enumerate the IFN-γ producing effector T cells. Here, the PBMCs' stimulated with Ag85A protein would be expected to generate CD4+ response, and the CFP-10 peptide (based on HLA-B*44), would be expected to generate a CD8+ T cell specific response. At the basal mRNA level we observed that the IFN-γ levels were significantly higher in

patients and contacts as compared to controls (Fig.2.7). Following Ag85A stimulation (CD4+ response) as well as CFP-10 peptide (CD8+ response) in ELISPOT assay, the spot forming units (SFU) were significantly increased in patients as compared to contacts (Ag85A: p= 0.03; CFP-10: p=0.003) and control (Ag85A: p= 0.012; CFP-10: p=0.0376).The difference between Ag85A and CFP-10 peptide stimulation was not very significant. (p=0.117) (Fig.2.11). Hence, in our study IFN-γ production was not found to be compromised at the basal level or following Mtb specific stimulation. These results are in accordance to Handzel et al.(2007) who in their study found no defect in IFNγ production in the serum or following stimulation of PPD with the PBMCs‟ of tuberculosis patients. Demissie et al. (2006) however, did not find any significant did not observe any significant difference in the IFN-γ mRNA levels of tuberculosis patients, contacts or control unstimulated leucocytes. Similarly, Demissie et al. also observed that the IFN-γ levels were similar in both patients and HHC. Whereas Sahiratmadja et al. (2007) found increased IFN-γ levels in controls when compared to tuberculosis patients, the levels of which reverted to control levels following anti-tubercular treatment. Hence different studies have shown variable results in case of IFN-γ production. In the present study we observed high levels of IFN-γ was being produced in patients in response to *M. tuberculosis* Ag/peptide.

Clinical studies have shown the correlation of TNF-blockers with progression from latent tuberculosis infection to disease (Harris and Keane , 2010). In the present study TNF-α mRNA levels were also found to be significantly high in patients and contacts compared to controls (Fig.2.6 and 2.7). Following stimulation with Ag85A and CFP-10 peptide the TNF-α mRNA level of patients and contacts was found to be variable and did not show any significant difference between the two groups (Fig. 2.9 and 2.11). Similarly Hirsch et al. (1996) in their study did not observe any significant difference in TNF-α levels following stimulation with PPD or 30 kda antigen. Contrastingly, Caccamo et al. have observed consistently high percentage of CD4+ T cells expressing IFN-γ/IL-2/TNF-α in active TB patients following Ag 85A/B stimulation (Caccamo et al., 2010).

Guyot-Revol et al. (2005) were the first to demonstrate the increased levels of CD4+ CD25 (high) FoxP3+ in active tuberculosis patients in blood and disease site in comparison to controls and emphasized on its importance in depressing the protective immune response to the disease. In the present study, we observed significant difference in the basal level FoxP3 expression of tuberculosis patients, HHC or controls (Fig 2.6 and 2.7). FoxP3 mRNA levels were highest in patients followed by HHC and controls. While most of the studies focused on CD4+ FoxP3+ T cells for studying Treg in tuberculosis patients, we evaluated the change in mRNA expression of FoxP3 following stimulation with a Th specific antigen and a CTL specific peptide. We observed that following Ag85A as well as CFP-10 peptide stimulation, significantly increased FoxP3 mRNA expression was observed in patients but not in contacts (Fig 2.10 and 2.11). In accordance to our study, Singh et al. (2012) observed a significantly increased population of CD4+Tregs among the CD4+ effector T cells in tuberculosis patients compared to healthy controls. Burl et al. (2007) also observed high levels of FoxP3 in blood samples of tuberculosis patients compared to healthy individuals. They noted that recently infected tuberculosis contacts had significantly low levels of FoxP3.

The role of Tregs in immunity to tuberculosis has not be completely deciphered. However, the production of IL-10 by Th2 and Tregs has been reported to be one of the major mechanisms leading to immunosuppressive response (Bonecini-Almeida et al., 2004; Ehlers, 1999; Singh 2012). In the present study, the basal mRNA levels of IL-10 were significantly higher in active tuberculosis patients as compared to contacts and controls (Fig 2.6 and 2.7). IL-10 levels were also significantly increased following Mtb specific antigen/peptide stimulation in patients but not in controls(Fig 2.10 and 2.11). Regarding, TGF-β mRNA levels were significantly higher in patients than contacts. The TGF- β mRNA levels increased following Ag85A stimulation but not following CFP-10 peptide stimulation in active tuberculosis patients indicating involvement of CD4+ T cells. In contrast, TGF-β mRNA levels did not increase following stimulation (Ag85A or CFP-10 peptide) in contacts(Fig 2.10 and 2.11). Olobo et al. (2001) in their study observed significantly increased plasma levels of TGF-β in active tuberculosis patients as compared to contacts however the plasma levels of IL-10 were not very different in

patients and contacts. The studies based on neutralizing antibody to TGF-β and IL-10 gave contrasting results. Hirsch et al. (1996) in their study reported increased IFN-γ production in response to PPD stimulated PBMCs of tuberculosis patients in the presence of anti-TGF-β and anti-IL-10 antibodies respectively. Contrastingly, Hougardy et al. (2007) observed that Mtb antigen (HAHB) stimulation of PBMCs of tuberculosis patients in the presence of anti-TGF-β and anti-IL-10 antibodies did not increase IFN-γ production. However, in the same study they observed increased IFN-γ levels when FoxP3+ T cells were depleted. Hence, besides IL-10 and TGF-β the role of Treg cells in immunosuppressive response is substantiated.

In summary, the study highlights certain crucial aspects correlating the pro- and anti-inflammatory cytokines with the immune status in tuberculosis patients, HHC and healthy controls. Firstly, FoxP3 mRNA expression clearly demarcates the three groups. Secondly, both CD4+ and CD8+ Treg cells were found to increase in patients compared to controls following *M. tuberculosis* specific antigen/peptide stimulation. These findings substantiate the role of Tregs as an important biomarker for immune status in tuberculosis infection. Thirdly, IFN-γ levels were found to be increased in patients at the basal level as well as following Mtb specific. These findings suggest that the tuberculosis patients are not compromised in their ability to produce IFN-γ but the increased levels of anti-inflammatory cytokines like IL-10 and TGF- β and increased Treg cells play important role in dampening the protective immune response.

CHAPTER 3

IN VITRO MULTINUCLEATE GIANT CELL FORMATION FROM MONOCYTES OF TUBERCULOSIS PATIENTS AND HEALTHY CONTROLS

"All progress is based upon a universal innate desire on the part of every organism to live beyond its income"

William Kingdon Clifford

CHAPTER 3

IN VITRO MULTINUCLEATE GIANT CELL FORMATION FROM MONOCYTES OF TUBERCULOSIS PATIENTS AND HEALTHY CONTROLS

3.1 INTRODUCTION

Tuberculosis begins with the inhalation of *M. tuberculosis* bacilli, which is primarily enocountered by alveolar macrophages. The early events taking place during infection are not clearly understood, although it is believed that alveolar macrophages kill most of the bacteria. Those bacteria that manage to survive, cross the lung parenchyma and reache the interstitial space where they get phagocytosed by the alveolar macrophages and cross the epithelial layer into the lungs. Once there, following the elaboration of various cytokines and chemokines, recruitment of monocytes and lymphocytes takes place leading to the formation of a granulomatous structure which is the hallmark of TB infection. The subsequent outcome of disease is however highly varied. The infecting bacteria may proliferate and spread leading to a progressive granulomatous disease (Davis and Ramakrishnan, 2009). Alternatively, it may be resolved following either an innate immune response or an adaptive immune response. However, in more than one-third cases, bacteria may persist giving rise to asymptomatic infection. A significant percentage of this population progresses to active disease without any discernable alteration in the immune response. It therefore appears that the outcome of infection is established by events taking place at this site of infection i.e. the granuloma. Granulomas are pathologic hallmarks of tuberculosis. The fate of the granuloma differs remarkably in an immunocompetent person in whom it undergoes calcification and eventually heals, as compared to an immunodeficient person where it leads to necrosis, cavitation and thereby spread of the disease (Sasindran and Torrelles, 2011). Thus, on one hand, granuloma seems to serve as a shelter for harboring the bacteria but on the other hand, the T cell mediated activation in the granuloma results in bactericidal or bacterio-static effect on the tubercle bacilli (Ehlers et al., 2010).

The major events in the process of granuloma formation are governed by the cytokines released by the immune cells in the granuloma, especially T cells. During the early events of infection, the dendritic cells which encounter the bacteria get activated and drain into the regional lymph node and by the virtue of their antigen presentation, stimulate the naïve T cells. The T cells take at least two weeks to be primed following which they arrive at the inflammation site and surround the lesion. Activated T cells arrive at the granulomas and move throughout the lesion in a constant motion. Each T cells probably makes a direct contact with most of the macrophages while wandering through the entire granuloma. However the most striking feature of granuloma is that despite this dynamic behavior the T cells are efficiently retained in the granuloma. Thus, within the granuloma the macrophages and the blood derived monocytes form a relatively immobile cellular matrix that interact with the highly dynamic population of effector T cells. The macrophages thereby act as scaffold for lymphocytes to crawl (Stoll et al., 2002).

3.1.1 Role of cytokines in granuloma

The interaction of T cells with macrophages leads to induction of several cytokines, some of which may be protective while others may contribute to the pathology of the disease. Several studies have indicated the importance of a balance between Th1 (T helper cell 1) cytokines and Th2 (T helper cell 2) cytokines in the pathology of tuberculosis (Cooper and Khader, 2008; Ramakrishnan, 2012; Almeida et al., 2009; Cavalcanti et al., 2012). Interleukin-2 which is a Th1 cytokine facilitates T cell replication and promotes cellular immunity apart from being a crucial factor for granuloma formation (Millington et al., 2007). Conversely, Th2 cytokine IL-10 inhibits T cell proliferation by down regulating the production of IL-2 (de Waal Malefyt et al., 1993). IL-10 is also known to contribute significantly to formation of a disorganized granuloma (Sasindran and Torrelles, 2011; Shaler et al., 2011). Several studies have demonstrated the immunosuppressive role of IL-10 in human and animal models (Cyktor et al., 2013; Beamer et al., 2008; Redford et al., 2010). Murine studies have demonstrated the role of IL-10 in reactivation as well as disease progression. Experimental evidence also suggests that use of IL-10 specific neutralizing antibody resulted in enhancement of tuberculosis proliferation (Zhang et al.,

1994). IL-4 is another TH2 cytokine that has been shown by various studies to be involved in MGC formation (Binder et al., 2013; McNally et al., 2011). In the present study, the role of IL-10 in MGC formation in the continued presence of IL-4, is described for the first time.

Among other cytokines, TNF-α also has a critical role in the maintenance of the granuloma and the formation of reactive nitrogen intermediates (RNI) that are formed in the activated macrophages (Cavalcanti et al, 2012; Ehlers et al., 2005). Using cytokine specific monoclonal antibodies against TNF-α, a 5-10 fold increase in reactivation of tuberculosis was observed (Ehlers et al., 2005). Additionally TGF-β also has been found to oppose the action of TNF-α thus contributing to the pathology of the disease (Aung et al., 2000; Allen et al., 2004).

3.1.2 Multinucleate giant cells

Based on the studies with granulomas formed in animal and human models, as well as from in vitro studies, some of the features that are characteristic of granulomas and the multinucleated giant cells (MGCs) formed within, show significant variation depending on the source and circumstances of granuloma development. For example, the Langhans cells commonly seen in tuberculous patients have several nuclei located towards the periphery surrounding the Golgi and other organelles. Unlike these, the foreign body giant cells have numerous nuclei (100-200) that are randomly distributed throughout the cytoplasm. Although the osteoclasts look similar to the foreign body giant cells, they have fewer nuclei dispersed throughout the cytoplasm. Foam cells on the other hand are characterised by multiple nuclei that are clustered in the middle and surrounded by foamy cytoplasm containing cholesterol and lipid droplets. As with morphology of the MGCs, studies have demonstrated differences in the importance of various cytokines in granuloma formation (Quinn and Schepetkin, 2009). However, the factors and mechanisms involved in the formation of MGC are not clear.

In a study conducted by Lay and co workers (2007), using PBMCs from non-tuberculous control donors, it was seen that virulent strains such as *M. tuberculosis* induces large multinucleated giant cells with more than 15 nuclei per cell, whereas the less virulent strains such as *M. avium* and *M. smegmatis* resulted in generation of MGCs having less than 7 nuclei per cell. Furthermore, the MGCs generated by *M. avium* and *M. smegmatis* retained their phagocytic ability while those by *M. tuberculosis* had lost their phagocytic ability. The study however did not look at the ability of PBMCs from tuberculous patients or house hold contacts of such patients. In these studies granuloma induction was either using mycobacterial antigen coated sepharose beads, or live mycobacterial.

A similar study with PBMCs from non-tuberculous control donors was also carried out to determine the effect of various mycobacterial lipid antigens on in vitro granuloma formation (Puiseggur et al., 2007). The lipid antigens included the pro-inflammatory phosphatidyl-myo-inositol mannosides and lipomannans (LM) which were able to induce granulomas and MGC formation, while the anti-inflammatory lipoarabino mannans formed granulomas without the concommittant MGC formation. Furthermore, only the LMs could induce large MGCs as seen within the granulomas of tuberculous patients. It would be interesting to see whether tuberculous patients have such soluble mediators circulating in their blood stream, and what effect they have on granuloma formation.

The histology of granuloma has traditionally been associated with type-1 cytokine pathway deficiencies. This type of genetic defect is known as mendelian susceptibility to mycobacterial infection. Studies have evidenced the strong association of deficiencies in IL-12p40, IL-12Rß1 or IFN-γ deficiency with disseminated infection with BCG or nonvirulent mycobacteria like *M.avium* (Fiechi et al., 2003; Arend et al., 2001). Emile et al., (1997) classified the granuloma on the basis of their structure into two types: 1) ‘tuberculoid granulomata’ which are well circumscribed well differentiated granulomas, characterized by the presence of epithelioid cells and multinucleated giant cells with very few or no bacteria (paucibacillary) and 2) ‘lepromatous-like granulomata’ which is a disorganized granuloma with very few giant cells or lymphocytes but numerous

macrophages infected with tubercle bacilli (multibacillary). Lammas et al. (2002) in their comparative study on biopsies from BCG infected patients with tuberculous granuloma and another group of patients with nontuberculous granuloma concluded that while the former type of granulomas were associated with increased survival, the later type indicated poor prognosis. Both the group of patients were receiving similar antitubercular chemotherapy. These findings imply that there might be a correlation between the multinucleate giant cell and the disease state of patients.

3.1.3 Models of granuloma study

While both mouse and human models of granuloma formation have been studied in the past, the observations and conclusions are based on circumstantial evidence. These approaches are also handicapped by the fact that only differentiated granulomas are accessible since it is not possible to study the various stages, and the physiological changes therein, leading to the development of the final granulomatous lesions. Therefore, more recently, in vitro studies have been carried out to get a better understanding of this aspect of TB. Recently, a new model which includes *M. marinum* and zebrafish was used to study the formation of fish granulomatous structures in real-time, to visualise the developments that take place during the initial course of infection, leading to granuloma formation. These structures correspond to the human granulomas and therefore provide an avenue for gaining better insight into the interaction that the bacilli have with the human host (Swaim et al., 2006).

In these studies, zebrafish were infected with *M. marinum* expressing recombinant fluorescent proteins allowing for the real-time study of infected and uninfected macrophages. Infected cells were responsible for further recruitment of uninfected phagocytes to the site of infection. Following apoptotic death of the former, they are phagocytosed by the uninfected macrophages, establishing a new growth niche for the pathogen and dissemination of the bacillus (Davis and Ramakrishnan, 2009; Swaim et al., 2006).

There have been other in vitro models developed with the help of PBMCs isolated from non-tuberculous controls and BCG vaccinated individuals, where certain macrophage functions have been looked at. These include induction of granulomas formation by different mycobacteria, as well as granuloma formation by different lipid antigens of MTB (Puiseggur et al., 2004). No studies however have been conducted using PBMCs from patients, or house hold contacts of patients, to understand the physiology of granuloma formation under these situations. Understanding the development and the physiological state of the granulomas in various situations, such as in patients versus contacts, before and after antibiotic treatment would give us a better insight into the disease.

From what is known in the literature, it can be be hypothesized that monocytes from patients and controls behave differently in response to cytokines produced by (patient or control) mononuclear cells following Mtb specific stimulation in vitro. To test this hypothesis, monocytes from patients and controls were incubated with culture supernatant of Con A and PPD stimulated mononuclear cells of both these groups. Subsequently, MGC formation was observed microscopically and along with this the various cytokine levels in culture supernatant were also analyzed. It was observed that patient monocytes behaved differently in the presence of cytokine containing supernatants from patients in comparison to healthy controls. Further, when the cytokine levels in culture supernatants were analysed it was found that IL-10 was consistently high in culture supernatants of patients than controls. To further investigate the role of IL-10 in MGC formation, IL-10 and IL-10 neutralizing antibody were also used with the culture supernatant reiterating the immunosuppressive role of IL-10 in tuberculosis granuloma.

In a separate experiment the blood samples of recently diagnosed active tuberculosis patients were taken before taking DOT (Directly observed therapy) and after two months of treatment. The MGC formation was observed in both the cases and cytokine analysis was done using cytokine bead assay. The MGC formation and cytokine levels were then compared to healthy controls.

3.2 MATERIALS AND METHODS

3.2.1 Study population

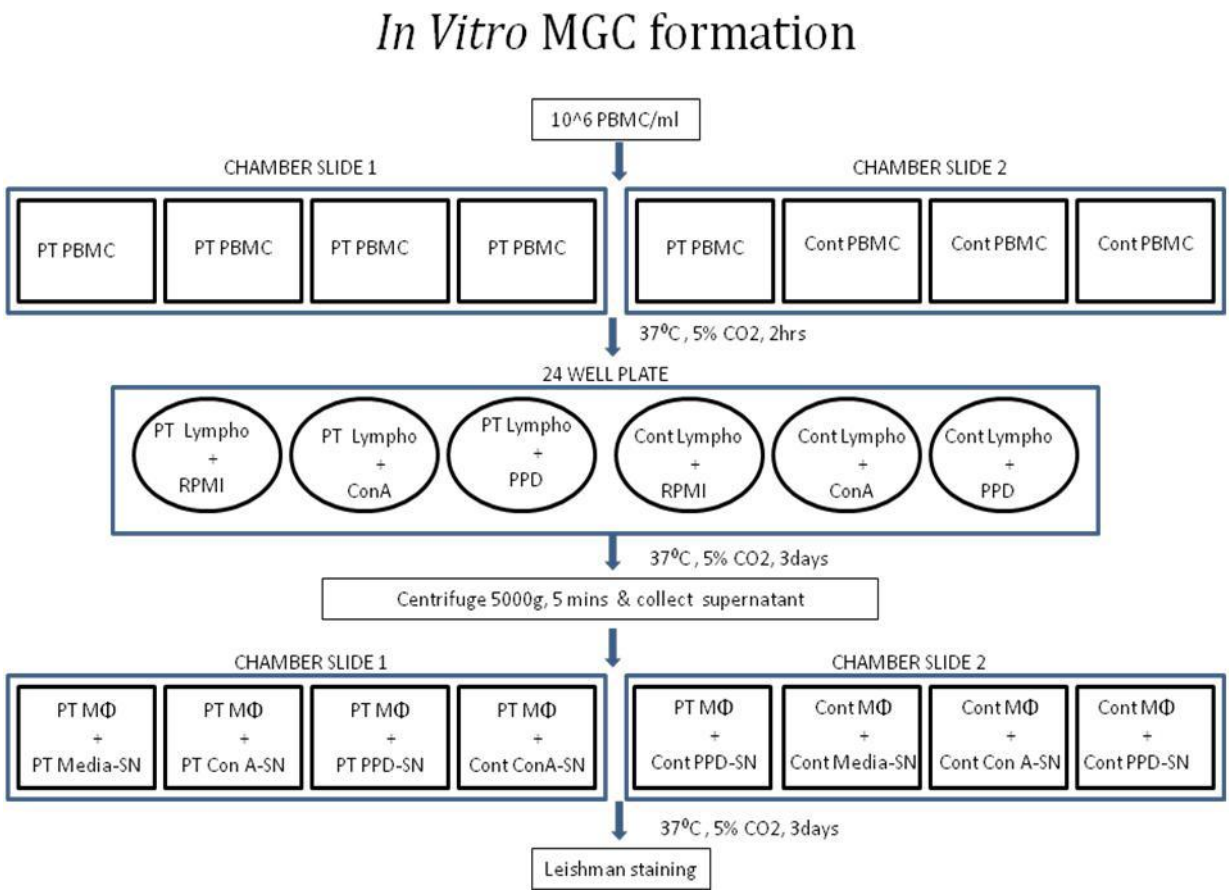
All patients (from SSG hospital, Vadodara) included in the study were newly diagnosed tuberculosis patients with roentgenographic findings (chest X-ray) consistent with TB and sputum positive for AFB (acid fast bacilli) and were human immunodeficiency virus negative. Asymptomatic healthy controls were also included in this study. Six males and four females of 20 to 50 years of age were included in each group. Blood samples were collected in EDTA vaccutainer. All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S. University of Baroda. Written informed consent was obtained from all subjects. In another experiment, the recently diagnosed active tuberculosis, HIV negative, patients were included. The blood samples were collected before and after two months of DOT treatment.

3.2.2 In vitro MGC formation

PBMCs were isolated from blood samples as described previously (Chapter 2 Materials and methods). Briefly, 10^6 PBMCs were seeded onto four well LabTek chamber slides (Nunc). Following two hours at 37°C and 5% CO2 the unadhered population was removed by repeated vigorous washing with RPMI 1640 culture medium. Subsequently, 2 X 10^5 cells of the above collected unadhered population of patients as well as healthy controls were seeded in 24 well plates respectively. Over 90% of the adherent cells were identified as monocytes by morphological criteria and fluorescence-activated cell sorting (FACS) analysis for the presence of CD14. Trypan blue dye exclusion was used to determine viability. The unadhered peripheral mononuclear cells were used as the source of lymphocytes and were stimulated for 3 days with 16µg/ml Concanavalin A (Con A) or 10µg/ml purified protein derivative (PPD, a kind gift from Kris Huygen, O.D. Communicable and Infectious Diseases, Brussels). The unstimulated cells of patients and healthy controls were taken as negative control. The supernatant from each of these wells were collected and then added at 50% final concentration to various monocyte cultures.

The supernatant obtained from healthy control unstimulated cells (C Med-SN) and those stimulated with Con A (C Con A-SN) and PPD (C PPD-SN) were then incubated with autologous healthy control as well as patient monocytes respectively. Also the supernatant obtained from patient unstimulated cells (Pt Med-SN) and those stimulated with both Con A (Pt Con A -SN) and PPD (Pt PPD-SN) were incubated with patient monocytes. Following incubation for 3 days, the monocytes were stained using Leishman stain and microscopic examination was carried out to study the size and fusion rate of MGC formation. The fusion rate was calculated as the number of nuclei within MGC (more than two nuclei per cell) in a given area per total number of nuclei in that same area:

Fusion rate (%) = (number of nuclei within MGC/total number of nuclei counted) X 100 (Gasser A, 1999). From each preparation 100-150 cells were selected from representative fields for calculation of fusion rate.



3.2.3 Cytokine ELISA

Cell culture supernatants were stored at -20^{0}C until analyzed. The levels of cytokines IL-2, IL-10, TNF-α and TGF-β was analyzed using sandwich ELISA immunoassay kits from GE Healthcare as per the manufacturer's instructions. IL-4 was analysed using commercially available ELISA kit (PeproTech EC Ltd,. London, UK).The absorbance was measured at 450 nm (IL-2, IL-10, TNF-α, TGF-β) and 405nm (IL-4) respectively and compared with the respective standard curve of the cytokines.

Briefly, for IL-2, IL-10, TNF-α and TGF-β, the microwell plate provided in the kit was precoated with monoclonal coating antibody of respective cytokines along with biotin-conjugate (polyclonal secondary antibody) and Streptavidin-HRP. Microwells containing standards of cytokines for various concentrations along with a blank well was also provided with the kit. 150µl of distilled water was added to the standard and blank wells.

All culture supernatants were analyzed directly except for TGF-β, for which the samples were diluted ten folds with sample diluent i.e. 180µl of Sample diluents + 20 µl sample. 20µl 1N HCl was added to the prediluted sample, incubated for 1 hour and then neutralized by addition of 20µl of 1N NaOH. 100µl of distilled water and 50 µl of culture supernatant was added to all the sample wells. In case of TGF-β 110µl distilled water and 40 µl of pretreated sample was added. The plates were covered with adhesive film and incubated for 3 hours on a microplate shaker at 200 rpm following which the plates were washed six times with 400 µl of wash buffer provided in the kit. The microwells were then developed by addition of TMB substrate solution to all wells including blank wells. The strips were incubated at room temperature (25°C) for 15 mins in dark. 100 µl

Stop solution was added to stop the reaction. The absorbance was measured at 450 nm and compared with the respective standard curve of the cytokines.

For IL-4, the microwell plate was coated overnight with anti-IL-4 antibody. The plate was then washed with wash buffer (PBS + 0.05% Tween-20) four times. The wells were then blocked with 1% BSA + PBS for an hour and washed similarly. The respective culture supernatant of 100µl was added to each well. The standards were diluted with PBS + 0.05% Tween-20 + 0.01% BSA and added to separate wells. Each sample was

added in triplicate. The plate was incubated at room temperature for two hours following which the plate was washed four times with wash buffer. This was followed by incubation with biotinylated secondary antiIL-4 antibody and then with avidin-HRP conjuagate for one hour each with intermittent washing four times with wash buffer. The substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS was then added and the plate was incubated at room temperature for colour development. The plate was read with 405nm and standard curve was prepared.

3.2.4 Effect of IL-10 and anti-IL-10 on MGC formation

For determining the role of IL-10 in MGC formation, IL-10 (50 ng/mL) (Peprotech, USA) as well as anti-IL-10 (5 μg/mL) (α-IL-10, Peprotech, USA) neutralizing antibody was used. Blood samples from healthy controls were taken (n=5) and in vitro MGC formation was observed as described above in 8 well Lab-tek chamber slide. The monocytes were incubated with autologous control culture supernatant having autologous Med-SN, Med-SN + IL-10, ConA-SN, ConA-SN + IL-10, ConA-SN + αIL-10 and ConA-SN + IL-10 + αIL-10. The fusion rate was observed following three days of incubation and staining the slides with Leishman stain.

3.2.5 Effect of IL-4 and anti-IL-4 on MGC formation

The effect of IL-4 was also studied in the same manner, using IL-4 (20ng/ml) and anti-IL4 (5 μg/mL). The monocytes obtained from five healthy controls were incubated with autologous control culture supernatant having autologous Med-SN, Med-SN + IL-4, Med-SN + IL-4 + αIL-4, ConA-SN, ConA-SN + IL-4, ConA-SN + αIL-4 and ConA-SN + IL-4 + αIL-4. The fusion rate was observed following three days of incubation and staining the slides with Leishman stain.

3.2.6 Cytokine analysis of culture supernatant of patients before and after treatment

Blood samples of active tuberculosis patients and healthy controls were collected before and after two months of treatment. The in vitro MGC formation was carried out as mentioned above. The culture supernatant of controls (C Med-SN, C Con-A-SN, C PPD-SN) patients before treatment (Pt Med-SN, Pt Con-SN, Pt PPD-SN) and after treatment

(TPt Med-SN, TPt Con-SN, TPt PPD-SN) were stored at -80°C until their cytokine levels were analysed. The concentrations of IL-2, IFN-γ, TNF-α, IL-10 and TGF-β were analyzed. The cytokine levels of the culture supernatant was then quantified using cytometric bead array (CBA) as per the manufacturer's (BD Biosciences, USA) instructions (Figure 3.1).

Briefly, the culture supernatant samples were added directly for all the cytokines except for TGF-β samples that were first activated using 1N HCL and then neutralized with 1.2 NaOH/ 1M HEPES. Except for TGF- β1 the lyophilized standard of all the cytokines were serially diluted in 1:2 to 1:256 ratio. The standard for TGF- β1 was diluted separately in the same manner and ratio. 50µl of the samples and standards were added to each tube. 50µl samples were added to each assay tube along with 50µl of capture bead and incubated for 1 hr in the dark except for TGF- β1 which was kept for 2 hours. Also TGF- β1 samples were washed at this step with 1 ml wash buffer provided in the kit. PE detection reagent was added to all the tubes and incubated for 2 hours. All the assay tubes were then washed with 1 ml wash buffer. The samples were then analysed using BD FACSAria^{TM} platform (FACSCalibur, BD Company, San Diego, CA). The minimum detection limits of these kits were 0.13pg/ml for IL-10, 0.8 pg/ml for IFN-γ, 1.2 pg/ml for TNF-α, 11.2 pg/ml for IL-2 and 14.9 for TGF-β. The data were analyzed by FCAP Array Software (BD Biosciences, USA).

Statistical analysis Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). The Mann–Whitney U-test was applied for group differences. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups.

Cytometric Bead Array

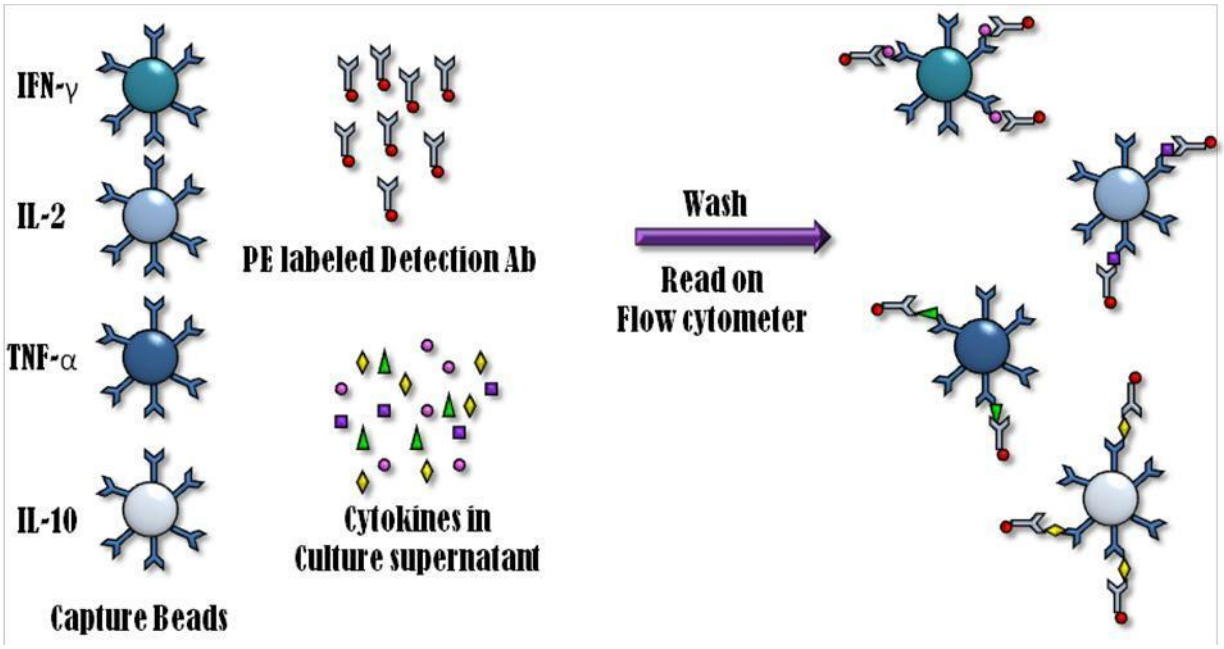


Figure 3.1: Scheme showing cytokine bead array analysis

3.3 RESULTS

3.3.1 *In vitro* MGC formation

The present study examined and compared the ability of patient and control monocytes to form MGC by treating them with cytokine containing supernatant of ConA or PPD treated nonadherent mononuclear cells. Figure 3.2 shows representative pictures of in vitro MGC formation by patient and control monocytes treated with various supernatants. The percent fusion rate was calculated as the ratio of the number of nuclei forming MGC to the number of total nuclei counted as given in Figure 3.3

As shown in Figure 3.2 and Figure 3.3, the monocytes of patients (Figure 3.2g) and controls (Figure 3.2h) incubated with their respective unstimulated supernatant of nonadherent mononuclear cells were found to be dispersed and very low fusion was observed (Control- 7.8%, Patient- 8.1%). However, the fusion rate of patient monocytes incubated with C ConA-SN (74.8%) (Figure 3.2b) was significantly higher ($p=0.0003$) than that with Pt ConA-SN (55.9%). (Figure 3.2a). Similarly, the fusion rate was significantly high ($p<0.0001$) in patient monocytes incubated with C PPD-SN (70.6%) (Figure 3.2e) than Pt PPD-SN (43.5 %) (Figure 3.2d).

It can also be seen from Figure 3.2 and Figure 3.3 that the control monocytes incubated with C ConA-SN (75.2%) and C PPD-SN (73.4%) had significantly higher ($p=0.0002$, $p< 0.0001$) fusion rates than patient monocytes incubate similarly with autologous supernatants respectively. However no significant difference was observed with patient or control monocytes when incubated with ConA-SN or PPD-SN derived from control peripheral mononuclear cells (Figure 3.2b, 3.2c, 3.2e, 3.2f).

3.3.2 Cytokine analysis of culture supernatant by ELISA

The levels of IL-2, TNF-α, IL-10 and TGF-β1 in patient and control supernatant were analysed by ELISA (Figure 3.4). IL-2 was significantly higher in C ConA-SN ($p=0.0016$) and C PPD-SN ($p=0.0025$) than Pt ConA-SN and Pt PPD-SN respectively (Figure 3.4a). Another Th1 cytokine, TNF-α however was not found to be significantly different in ConA-SN or PPD-SN of patients and controls (Figure 3.4b). Also, neither IL-2 nor TNF-

α, showed any difference in the spontaneous release without any antigen stimulation in both the groups.

IL-10, on the other hand showed significantly high levels in Pt ConA-SN ($p<0.0001$) and Pt PPD-SN ($p<0.0001$) compared to C ConA-SN and C PPD-SN respectively (Figure 3.4c). On the contrary the other Th2 cytokine TGF-β did not show any significant difference when Pt ConA-SN and Pt PPD-SN were compared to C ConA-SN and C PPD-SN respectively (Figure 3.4d). Although IL-10 levels in Med-SN between patients and controls did not show any significant difference, TGF-β1 levels were found to be significantly high ($p = 0.001$) in patients.

3.2.3 Role of IL-10 and IL-4 in *in vitro* MGC formation

In order to confirm the role of IL-10 in MGC formation, control monocytes were incubated independently with autologous Med-SN, Med-SN +IL-10, ConA-SN, ConA-SN + IL-10, ConA-SN + αIL-10 and ConA-SN + IL-10 + α IL-10 respectively. Figure 3.5 shows the fusion rates for these treatments. No significant difference in the fusion rate was observed when control monocytes were incubated with Med-SN (9.2 %) or Med-SN + IL-10 (7%). However, the fusion rates decreased significantly ($p<0.0001$) following treatment of monocytes with ConA-SN + IL-10 (50.4%) as compared to ConA-SN (81.8%). Although aggregates were observed in the former, the rate of fusion was significantly reduced compared to the latter. To further confirm the effect of IL-10, when IL-10 neutralizing antibody was used, it was found that the rate of fusion in the presence of ConA-SN containing both IL-10 and neutralizing antibody (73.6%) was significantly increased ($p=0.0004$) compared to ConA-SN+ IL-10 alone (50.4%). There was no significant effect observed when anti-IL-10 was included solely with ConA-SN (71.6%).

In contrast to IL-10, the levels of IL-4 were not found to be different in the culture supernatant of patients and controls whether or not they were stimulated by ConA or PPD (Figure 3.4). However, when IL-4 was added exogenously along with autologous Med-SN and ConA-SN to evaluate in vitro MGC formation, it was observed that the fusion rate was significantly increased in both the cases ($p= 0.0001$ and $p= 0.0037$ respectively)

(Figure 3.6). Furthermore, upon addition of IL-4 neutralizing antibody, this effect was reversed and significant decrease in the fusion rate was observed in both Med-SN (p= 0.0001) as well as Con-SN (p= 0.0003) (Figure 3.6).

3.3.4 Comparison of *in vitro* MGC formation in patients before and after treatment

In another experiment *in vitro* MGC formation was studied in five active tuberculosis patients before and after two months of treatment and compared with that in healthy controls for the same time period. All patients were showing good prognosis following treatment as indicated by chest X-ray and sputum AFB findings. The results obtained when active tuberculosis patients were compared with healthy controls were similar to that of the previous experiments. As shown in Fig.3.7 and Fig.3.8 it was observed that in active tuberculosis patients, the MGC formation and thereby the fusion observed following incubation with autologous P ConA-SN and P PPD-SN respectively were significantly ($p<0.05$) lower than that of controls . Also, as previously observed, when the patient macrophages were incubated with control culture supernatants (C ConA-SN and C PPD-SN). Surprisingly, it was observed that when *in vitro* MGC formation of the same patients was investigated after two months of treatment the rate of MGC formation significantly increased when patient monocytes were incubated with their autologous supernatants (P ConA-SN and P PPD-SN) respectively. All the more, the MGC formation observed in this case was similar to that observed when patient monocytes were incubated with control supernatants (C ConA-SN and C PPD-SN) respectively and when control monocytes were incubated with their autologous supernatants. This implies that the MGC forming ability of tuberculosis patients increased and reached to the levels similar to controls following anti-tuberculosis treatment (Fig 3.7 and 3.8).

3.2.5 Cytokine bead array analysis of patients' in vitro activated lymphocyte culture supernatant before and after treatment

The analysis of cytokines in culture supernatant of patients before and after anti-tuberculosis treatment was analysed using multiplex assays. These cytokines include IL-2, IFN-γ, TNF-α and IL-10. Another cytokine TGF-β was analyzed by single plex assay.

As shown in Fig.3.9A and B and Fig.3.14A and B the gating was done during FACS analysis and the standards and samples were analyzed. The data was evaluated and standard graphs (Fig.3.9C-F and Fig.3.14C) of the respective cytokines were prepared by FCAP Array Software (BD Biosciences, USA). The cytokine levels of IL-2 were significantly high ($p<0.05$) in C Med-SN than Pt Med-SN and TPt Med-SN. No difference in IL-2 levels was observed in culture supernatants following ConA stimulation. However there was a significant increase in the Mtb specific IL-2 levels in patients following treatment ($p<0.05$) as observed in PPD-SN. The levels of IL-2 were similar in C PPD-SN and TPt PPD-SN (Fig.3.10). In case of IFN-γ, the levels of IFN-γ in Pt PPD-SN were not found to be significantly different from C PPD-SN but were significantly lower than TPt PPD-SN. The rest of the supernatants in case of IFN-γ did not show any significant difference (Fig.3.11). The levels of TNF-α (Fig.3.12)and TGF-β (Fig.3.15) were also not found to be significantly different in patients, before and after treatment as well as controls. The IL-10 levels however were found to be significantly different in both PPD-SN and ConA-SN in patients before treatment in comparison to that of healthy control and patients after treatment and supernatants (Fig.3.13).

3.4 DISCUSSION

A comparison of MGC forming ability of tuberculosis patients and healthy controls using an in vitro model was carried out in this study. Several groups have used in vitro granuloma model in the past to gain better understanding of immunopathology of tuberculosis using monocytes of only healthy controls (Gasser and Most, 1999; Lay et al., 2007). Gasser and Most (1999) reported the in vitro differentiation of monocytes to MGC using mycobacteria in combination with BCG stimulated T cell supernatant of control lymphocytes. In another study, Lay et al. (2007) studied an in vitro granuloma model and reported that MGC formed in Mtb induced granuloma loose their ability of bacterial uptake but retain their bactericidal activity following MGC formation. However, no study appears to have been done to compare the monocytes of tuberculosis patients and healthy controls in their in vitro MGC forming ability with particular reference to the role of IL-10. In the present study, the differences in MGC formation of patient and control monocytes (adhered cells) when incubated with their respective culture supernatants obtained following ConA or PPD stimulation of autologous peripheral mononuclear cells (unadhered cells) was examined. MGC formation was also evaluated for patient monocytes incubated with ConA or PPD stimulated control peripheral mononuclear cell (unadhered cells) culture supernatant. It is observed that the rate of fusion and MGC formation in case of patient monocytes was much less than that of healthy controls when incubated with their autologous supernatant (Figure 3.2a,3.2d,3.2c,3.2f,3.3). Furthermore, when the same patient monocytes were incubated with ConA or PPD stimulated control peripheral mononuclear cell culture supernatant the rate of fusion was surprisingly increased (Figure 3.2b, 3.2e, 3.3). These results reflect that the patient monocytes have the ability to form MGC. However, the difference in the results obtained when patient monocytes are treated with patient supernatant or control supernatant reflects upon the difference in the cytokine produced by the two groups.

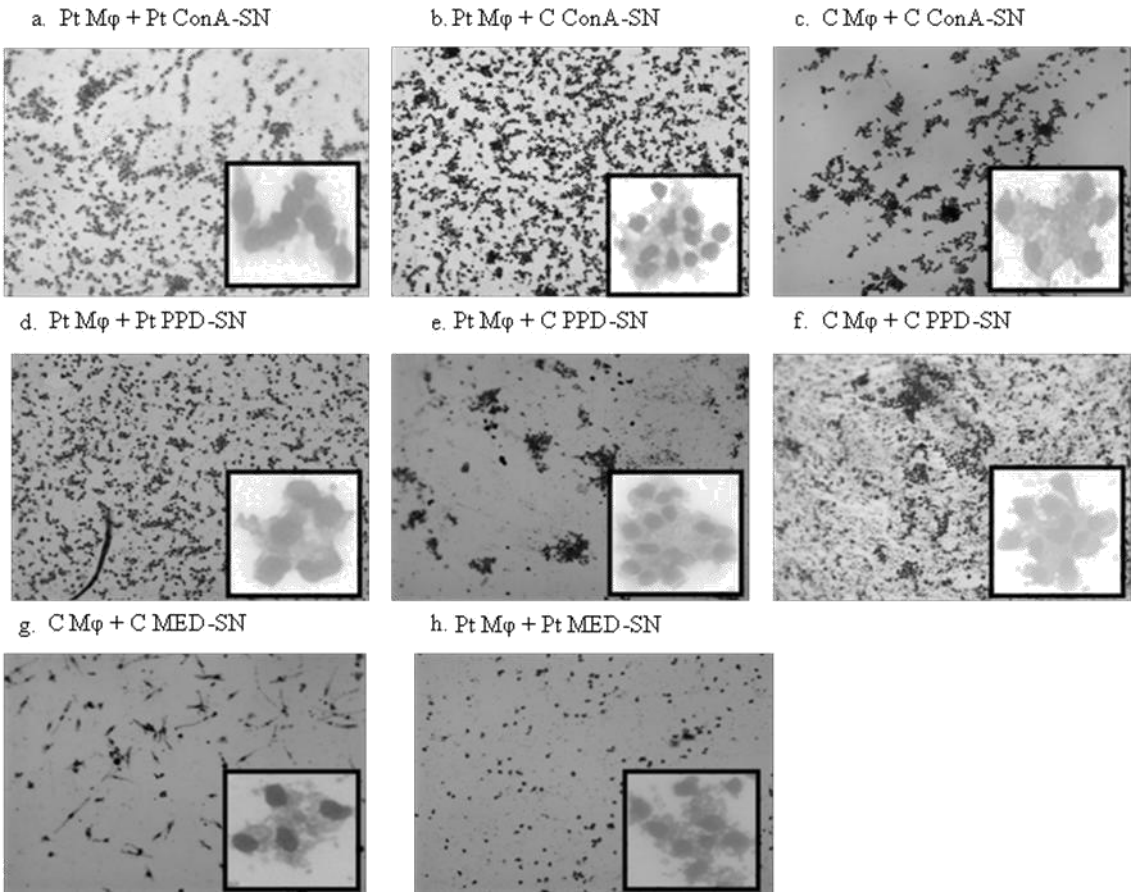


Figure 3.2 Leishman stained representative pictures of *in vitro* MGC. Monocytes from patients (Pt) and controls (C) were incubated with culture supernatant derived from unstimulated control (Med-SN), ConA stimulated (ConA-SN) and PPD (PPD-SN) stimulated peripheral mononuclear cells obtained from patients and controls respectively. Patient monocytes were incubated with Pt ConA-SN (a), C ConA-SN (b), Pt PPD-SN (d), C PPD-SN (e) and Pt Med-SN (h). Control monocytes were incubated with C ConA-SN (c), C PPD-SN (f) and C Med-SN (g)

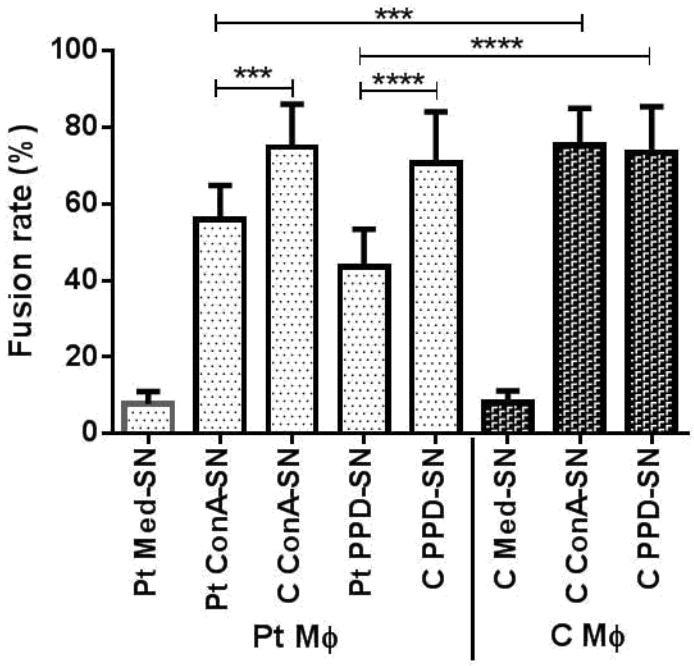


Figure 3.3 Graph showing percent fusion rate of in vitro MGC formation.

Monocytes from patients (Pt Mφ) (n=10) and controls (C Mφ) (n=10) were incubated with supernatant derived from unstimulated control (Med-SN), ConA stimulated (ConA-SN) and PPD stimulated (PPD-SN) peripheral mononuclear cells obtained from patients and controls respectively. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

Organized granuloma is a prerequisite to limit mycobacterial infection, the fate of which is decided by both Th1 as well as Th2 cytokines. Hence the IL-2, IL-4, TNF-α, TGF-β and IL-10 cytokine levels present in the respective lymphocyte supernatants of patient and healthy controls were investigated by ELISA. IL-2, which is a Th1 cytokine, contributes to cellular immunity by facilitating T cell replication and granuloma formation (Millington et al., 2007). IL-2 levels were found to be significantly increased in PPD stimulated lymphocytes of controls but not patients (Figure 3.4). This is in agreement with Birkness et al. who observed MGC formation in control PBMCs and macrophages following treatment with IL-2, although not including patient lymphocytes in their study (Birkness et al., 2007). However, contrary to the above two observations, Gasser and Most (1999) conclude from their study that there was no correlation between IL-2 levels and in vitro MGC formation. Therefore, it appears that in accordance with the observation of Birkness et al. (2007), in the present study, IL-2 which is produced in response to PPD contributes to the higher fusion rate seen with control PPD-SN compared to patient PPD-SN.

The role of TNF-α is quiet varied as demonstrated by many studies that have indicated that TNF-α did not have a role in MGC formation (Most et al., 1990; Takashima et al., 1993). Studies have also highlighted a role for TNF-α in MGC formation and maintenance of granuloma (Birkness et al., 2007). In the present study, variable levels of the cytokine following stimulation with ConA or PPD in case of both patients and control cells was observed (Figure 3.4). This indicates that TNF-α may not be absolutely essential for MGC formation while the presence of it does not interfere with MGC formation.

IL-4 is a Th2 cytokine which increases TNF-α toxicity thereby aggravating tissue damage and inducing fibrosis of granuloma leading to enhanced immunopathology (Rook et al., 2004). Several studies have acknowledged and demonstrated the role of IL-4 in MGC formation (Binder et al., 2013; McNally and Anderson, 2011). In addition, excessive IL-4 production has also been correlated with active TB and a depressed Th1 response

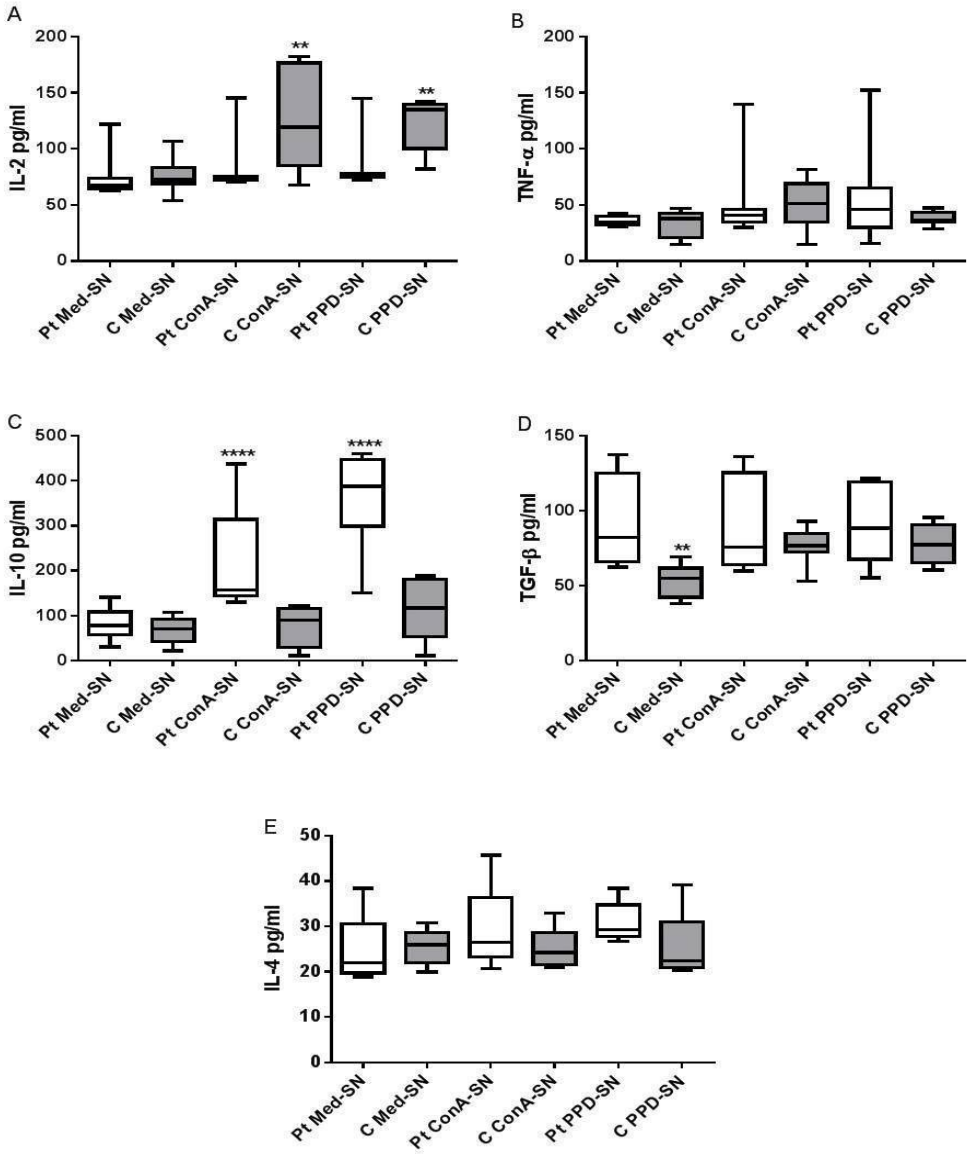


Figure 3.4 Cytokine analysis of culture supernatants. Culture supernatants obtained from peripheral mononuclear cells of patients (Pt) and controls (C) incubated with ConA (ConA-SN) and PPD (PPD-SN). Control wells were seeded with cells without any antigen/mitogen (Med-SN). The levels of (A) IL-2 , (B) TNF-α, (C) IL-10 (D) TGF-β and (E) IL-4 were analysed by ELISA. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

(Hernandez-Pando et al., 2004). However, as shown in Figure 3.4, the levels of IL-4 was found to be similar in both patients and controls whether or not they were stimulated with ConA or PPD.

IL-10 is a potent anti-inflammatory cytokine that deactivates macrophages, down regulates Th1 response and limits antigen presentation. High levels of IL-10 have been associated with disorganized granuloma formation (Sasindran and Torrelles, 2011). Significantly high levels of IL-10 in patient PPD-SN but not in case of control PPD-SN was observed (Figure 3.4). This implies that patients have higher population of T cells that can produce high levels of IL-10 compared to that of control lymphocytes. Similar observations were made by Pereira et al. (2004) where in case of both ex vivo and in vivo exposure of monocytes to Mtb, the IL-10 and TNF-α levels were significantly high in active tuberculosis patients compared to healthy controls.

TGF-β is known to alter the production and function of TNF-α (Aung et al., 2000; Allen et al., 2004). In addition, a negative correlation between TGF-β and IL-10 with organized granuloma formation has also been reported (Sasindran and Torrelles, 2011). Aung et al. (2000) correlated the presence of TGF-β1 in mononuclear phagocytes present in the lung lesion with granuloma formation. Othieno et al. (1999) in their study emphasized on the importance of synergistic action of IL-10 and TGF-β in down regulating IFN-γ production and thereby dampening the immune response to tuberculosis infection. Studies have also shown elevated levels of IL-10 (Lee et al., 2002) and TGF-β (Toosi et al., 1995) following stimulation of PBMC isolated from tuberculosis patient with PPD. In the present study however, insignificant change in the levels of TGF-β in stimulated cells in tuberculosis patients and controls was observed. In contrast, IL-10 which was found to be significantly high in comparison to unstimulated cells of tuberculosis patients but not in controls. The contrasting results obtained in case of TGF-β levels observed in the present study in comparison to other studies may be attributed to the population of cells used for study. While in the studies mentioned above PBMCs were used as a source of stimulation, in the present study unadhered cells were used as a source of lymphocytes. These unadhered cells are devoid of monocytes. As is evident from the literature

monocytes have been known to be the principle source of TGF-β (Ohtsuka et al., 1998). Also among the lymphocytes NK cells (Ohtsuka et al., 1998) and Tregs (Vernal and Garcia-Sanz, 2008) are known to produce TGF-β. However these cells constitute very small population of lymphocytes. This may explain the insignificant change in the levels of TGF-β in stimulated cells in contrast to IL-10 which was found to be significantly high. IL-10 is produced by B lymphocytes, dendritic cells, Th2 cells and Tregs.

Interestingly, Th2 cytokines IL-10 and IL-4 have opposite effects on MGC formation. On one hand IL-10 has been correlated with disorganized granuloma formation whereas IL-4 is known to favour MGC formation. In order to investigate which of the two Th2 cytokines has a predominant effect on MGC formation, monocytes were treated with exogenous cytokines in the presence and absence of their corresponding neutralizing antibodies. The results confirm the immune suppressive role of IL-10 wherein inclusion of IL-10 led to reduced MGC formation which was reversed by addition of anti-IL-10 antibody (Fig.3.5). Conversely, addition of IL-4 led to significant increase in MGC formation and the effect was neutralized following addition of anti-IL-4 antibody (Fig.3.6). These results therefore demonstrate that IL-10 appears to override the effect of IL-4 in patient supernatants which in turn is responsible for reduced MGC formation

In the present study, no significant difference in the levels of TGF-β1 following stimulation of patient and control mononuclear cells with ConA or PPD in comparison to their respective unstimulated culture supernatants was observed. Interestingly, TGF-β1 levels observed in unstimulated culture supernatants was significantly high in patients as compared to controls (Figure 3.4). Furthermore, this heightened level of TGF-β1 was not increased any further following stimulation with either ConA or PPD. Since, IL-10 and not TGF-β1 levels were significantly different in stimulated control and patient derived culture supernatants, the immunosuppressive effect of IL-10 by using IL-10 and IL-10 neutralizing antibody in the system was additionally studied. The results confirm the immune suppressive role of IL-10 wherein inclusion of IL-10 lead to reduced MGC formation. This effect was neutralized when anti-IL-10 antibody was included in the system. .

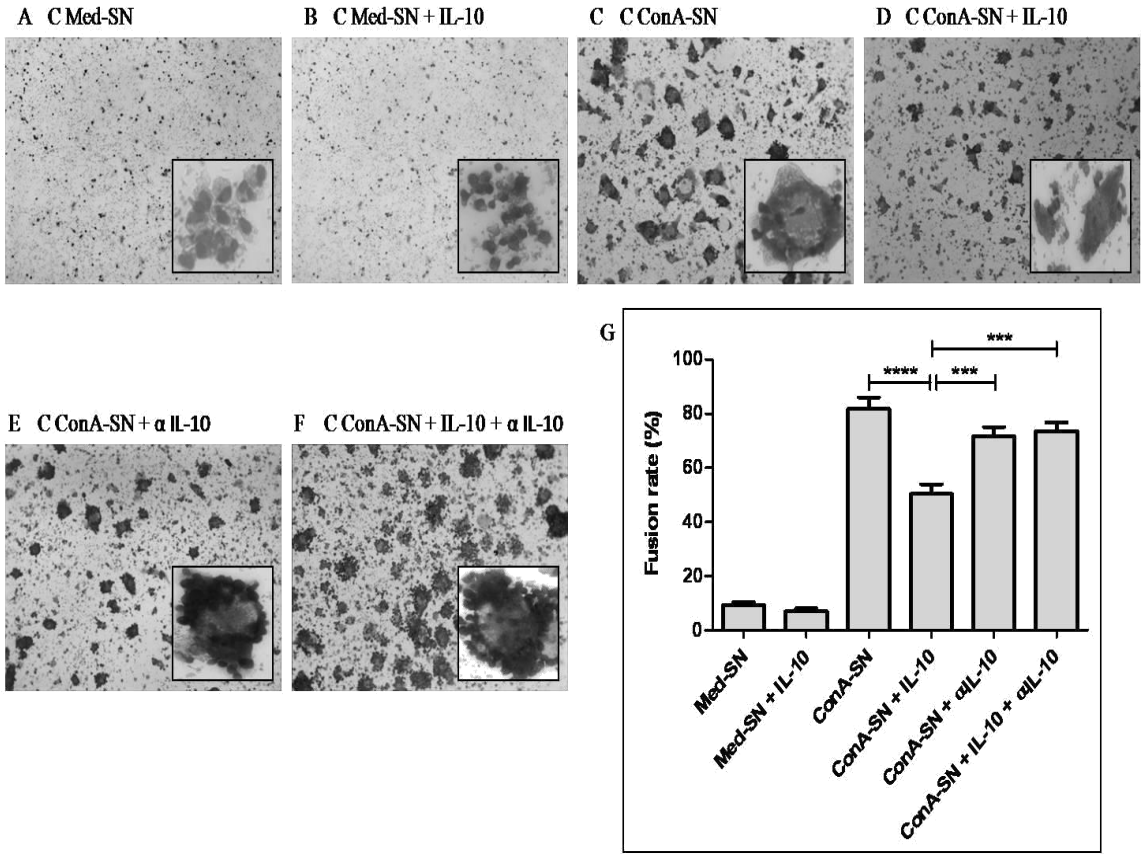


Figure 3.5 Effect of IL-10 and anti-IL-10 antibody (α IL-10) on in vitro MGC formation. Figure shows representative Leishman stained pictures of MGC formation and fusion rate of monocytes obtained from healthy controls (n=5) incubated with autologous culture supernatants. (A) unstimulated culture supernatant (Med-SN) (B) Med-SN + IL-10 (C) ConA stimulated culture supernatant (ConA-SN) (D) ConA-SN + IL-10 (E) ConA-SN + α IL-10 (F) ConA-SN + IL-10 + α IL-10 (G) Graph showing effect of IL-10 and anti-IL-10 on percent fusion rate of in vitro MGC formation. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

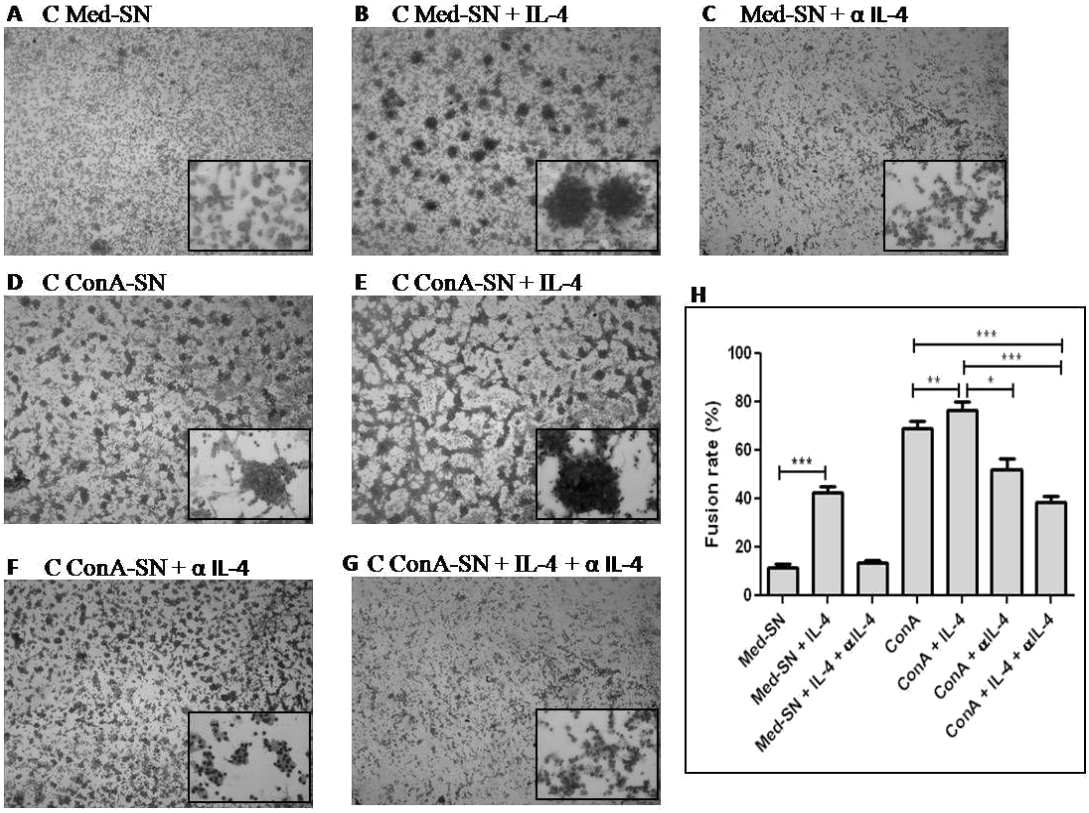


Figure 3.6 Effect of IL-4 and anti-IL-4 antibody (α IL-4) on in vitro MGC formation. Figure shows representative Leishman stained pictures of MGC formation and fusion rate of monocytes obtained from healthy controls (n=5) incubated with autologous culture supernatants. (A) unstimulated culture supernatant (Med-SN) (B) Med-SN + IL-4 (C) Med-SN + IL-4 + αIL-4 (D) ConA stimulated culture supernatant (ConA-SN) (E) ConA-SN + IL-4 (F) ConA-SN + α IL-4 (G) ConA-SN + IL-4 + α IL-4

(H) Graph showing effect of IL-4 and anti-IL-4 on percent fusion rate of in vitro MGC formation. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.000$

As evident from Figure 3.4 control culture supernatant stimulated with ConA as well as PPD contained high levels of IL-2. Despite the presence of IL-2, IL-10 cytokine was effective in reducing the fusion rates of control monocytes and hence the need to neutralize IL-2 to observe this effect was not felt necessary. Hence in the present study, decreased fusion rates were observed with exogenously added IL-10 despite the presence of IL-2 indicating that IL-2 did not or have very little role to play in opposing the effect of IL-10 in MGC formation.

This study demonstrates that patient and control monocyte have similar potential in MGC formation in vitro as evident from the fusion rates given in Figure 3.3. However, the cytokine producing ability of their lymphocytes differs with regard to both Th1 cytokine IL-2 and Th2 cytokines IL-4, IL-10 and TGF-β. The role of IL-4 in MGC formation was confirmed in the present study. In addition, we have also confirmed the immunosuppressive role of IL-10 in MGC formation in patients as compared to controls, despite similar levels of IL-4 found in both the groups. Hence, this study establishes the unimpaired potential of patient monocytes in MGC formation and that the apparent impaired functionality of patient monocytes in MGC formation is likely due to the presence of IL-10.

Following infection, the type of granuloma formed (tuberculoid or lepromatous) reflects on the immune status of the individual. The tuberculoid granuloma is well circumscribed and characterized by numerous epitheliod and MGCs whereas the lepromatous granuloma is disorganized and has very few MGCs (Emile et al., 1997). Lammas et al., (2002) observed that higher survival rate in BCG infected patients can be correlated with formation of tuberculoid granuloma whereas lepromatous granuloma were associated with poor prognosis of the disease. Hence, it can be hypothesized that the presence of MGC could be correlated with disease prognosis.

Therefore, in another experiment, the in vitro MGC forming ability of the patients was analyzed before and after treatment. The results of MGC formation in case of patients before treatment and controls were similar to that obtained in the previous experiment. It was however surprising to note that the MGC formation in patients increased

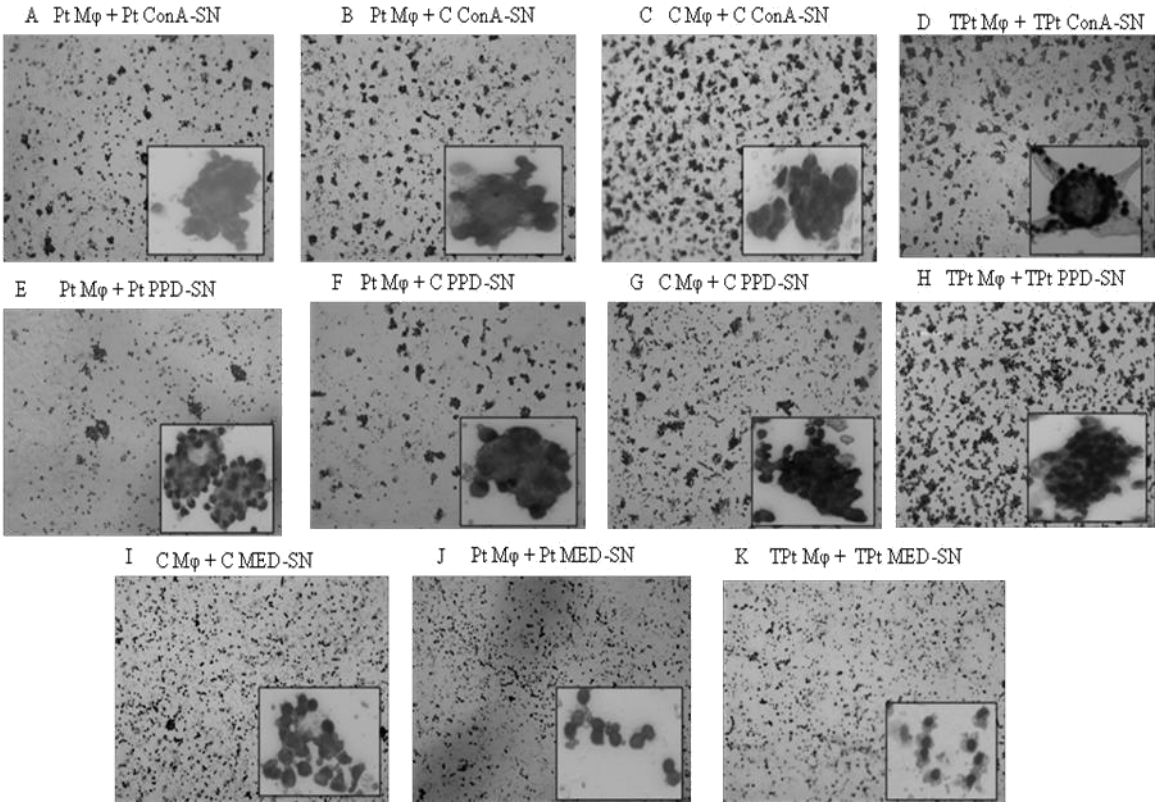


Figure 3.7 Leishman stained representative pictures of *in vitro* MGC of controls and patients before and after treatment. Monocytes from patients (Pt) and controls (C) and patients after two months of anti-tuberculosis treatment (TPt)were incubated with culture supernatant derived from unstimulated control (Med-SN), ConA stimulated (ConA-SN) and PPD (PPD-SN) stimulated peripheral mononuclear cells obtained from patients, controls and treated patients respectively. Patient monocytes were incubated with Pt ConA-SN (a), C ConA-SN (b), Pt PPD-SN (e), C PPD-SN (f) and Pt Med-SN (j). Control monocytes were incubated with C ConA-SN (c), C PPD-SN (g) and C Med-SN (i). Treated patient monocytes were incubated with TPt ConA-SN (d), TPt PPD-SN (g) and TPtMed-SN (e)

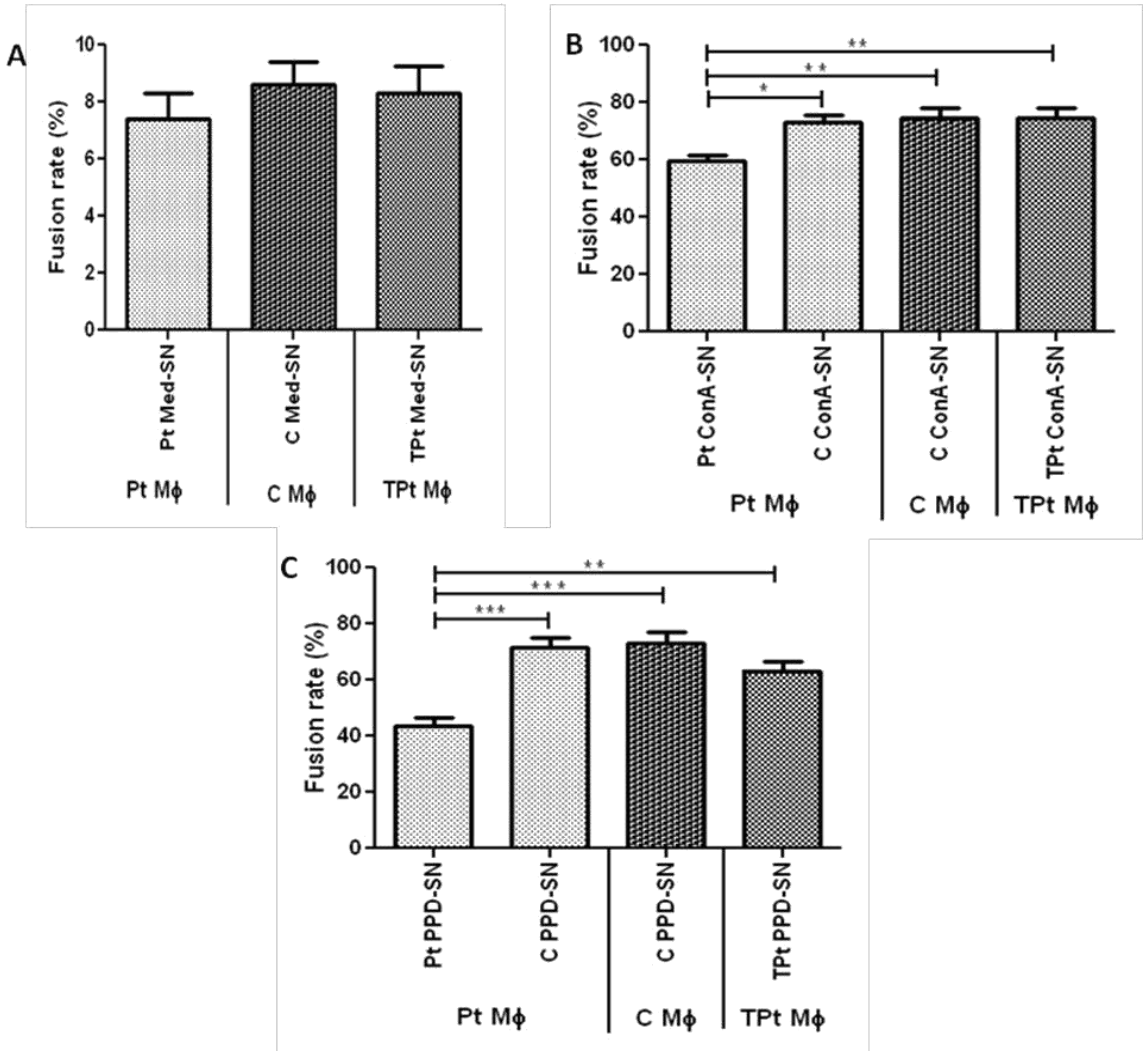


Figure 3.8 Graph showing percent fusion rate of in vitro MGC formation in patients before and after treatment and controls. Monocytes from patients (n=5) before (Pt Mφ) and after anti-TB treatment (TPt Mφ) and controls (C Mφ) (n=5) were incubated with supernatant derived from A) unstimulated control (Med-SN) , B) ConA stimulated (ConA-SN) and C) PPD stimulated (PPD-SN) peripheral mononuclear cells obtained from patients and controls respectively. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

significantly when patient monocytes were incubated with autologus ConA-SN and PPD-SN, following two months of treatment (Fig 3.7 and 3.8). It was therefore hypothesized that this change might be due to the difference in cytokine levels of both the groups. To test this hypothesis, cytokine bead array of culture supernatants used for MGC formation was performed. It was observed that the levels of IL-2 in Med-SN and PPD-SN were significantly raised in patient following two months of treatment (Fig. 3.10). This is in accordance with the results obtained by ELISA analysis in the previous experiment where *M. tuberculosis* specific release of IL-2 was significantly higher in patients than controls. Interestingly, the IL-2 levels of treated patients (P PPD-SN) reached levels similar to that controls (S-PPD). IFN-γ levels remained unchanged in case of unstimulated and ConA-stimulated culture supernatants of all the groups. However, the levels of IFN-γ in PPD-stimulated culture supernatants increased significantly in patients after therapy whereas those of controls did not show any significant difference from any of the patient PPD – SN (Fig. 3.11). Studies have shown that following treatment the T cell population changes from only IFN-γ producing and IL-2/ IFN-γ producing population to predominantly IL-2/ IFN-γ producing T cell population (Millington et al., 2007; Suter-Riniker et al., 2011) which is in agreement with the findings of this study. However, it is known that IL-2 does not confer any significant protection when given as adjunct therapy to drug sensitive tuberculosis patients. (Johnson et al., 2003).

In accordance to the results of TNF-α (Fig.3.12) and TGF-β (Fig.3.15) in the previous experiment, we did not observe any significant difference in these cytokines of any culture supernatants of patients before treatment in comparison to the control supernatants. The culture supernatants of patients after treatment also showed similar TNF-α and TGF-β levels to that of patients before treatment and healthy controls.

Following treatment the cytokine profiles in general tends to move more towards the control profile indicating a reversal of the pathophysiological state of the patients. As can be seen in the figures (Fig 3.9-3.15) all the peaks corresponding to the cytokines in treated patients shift to the position which is concomitant with that observed for controls. This reversal of cytokine secreting pattern has also been observed by others.

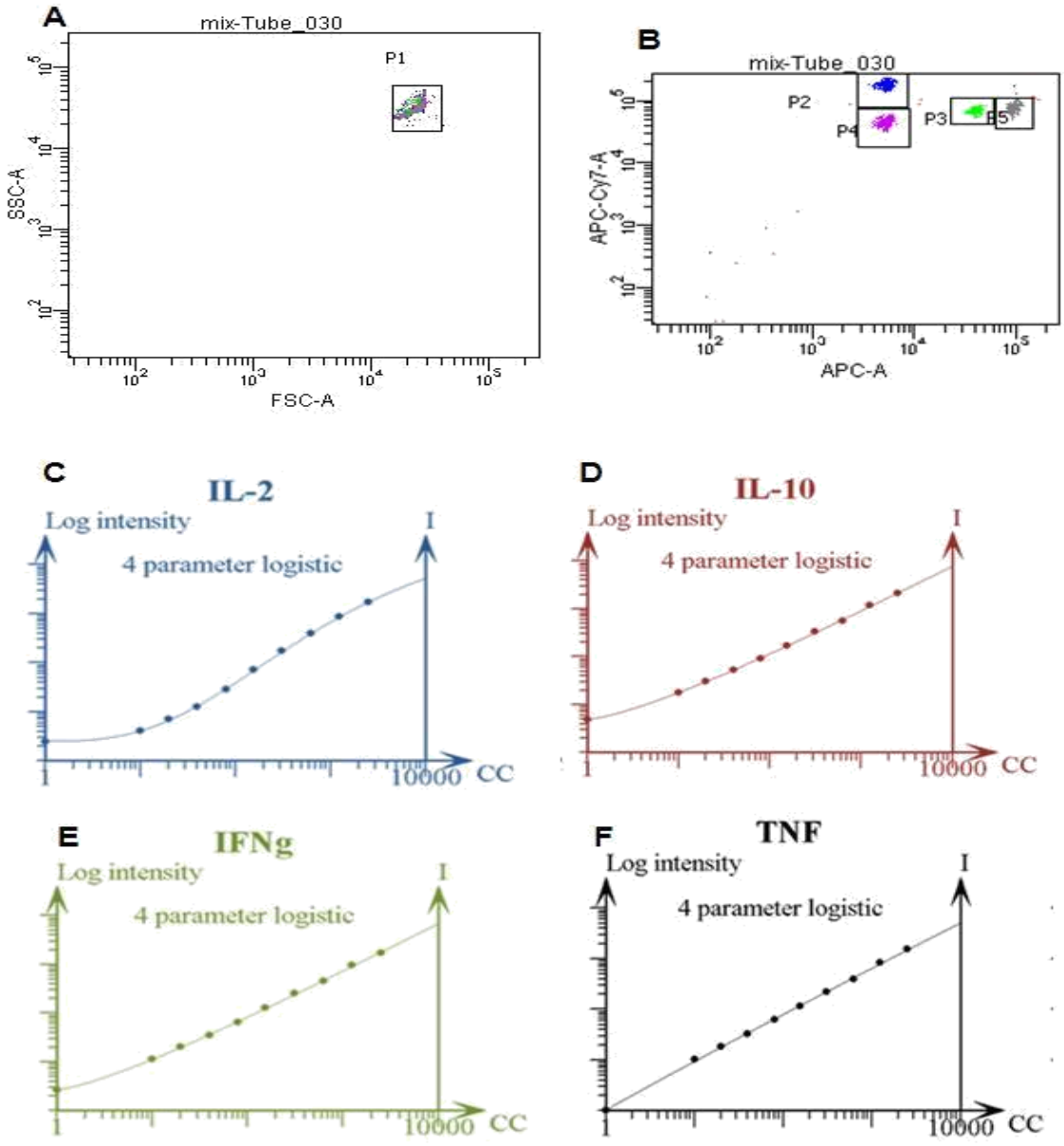


Figure 3.9 FACS plots showing gating and standard graphs of multiplex cytokine bead array. (A) Representative FACS plot showing parental population (P1) of cytokine beads (B) Representative FACS plots for IL-2 (P2) population at the A4 position (Blue), IFN-γ (P3) population at the B8 position (Green), IL-10 population at the B7 position (Pink), TNF-α (P4) at the C4 position (Grey). In addition, the representative standard graphs of (C) IL-2, (D) IL-10 and (E) IFN-γ and (F) TNF-α are also shown.

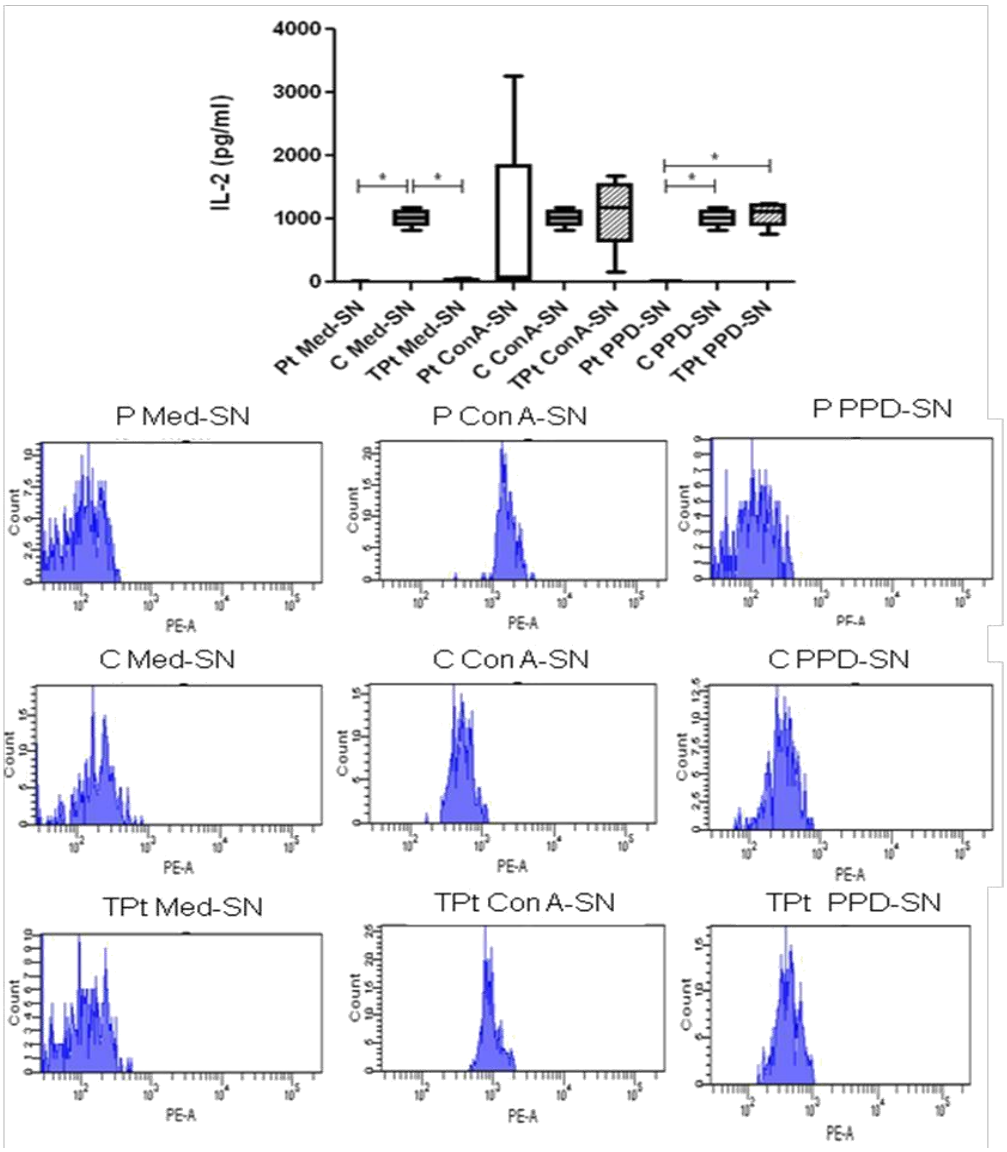


Figure 3.10 Graph and Histograms representing CBA Analysis of IL-2 in culture supernatants. Culture supernatants obtained from peripheral mononuclear cells of patients before (Pt) and after treatment (TPt) and controls (C) and incubated with ConA (ConA-SN) and PPD (PPD-SN). Control wells were seeded with cells without any antigen/mitogen (Med-SN). The levels of IL-2 were analysed by cytometric bead array. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

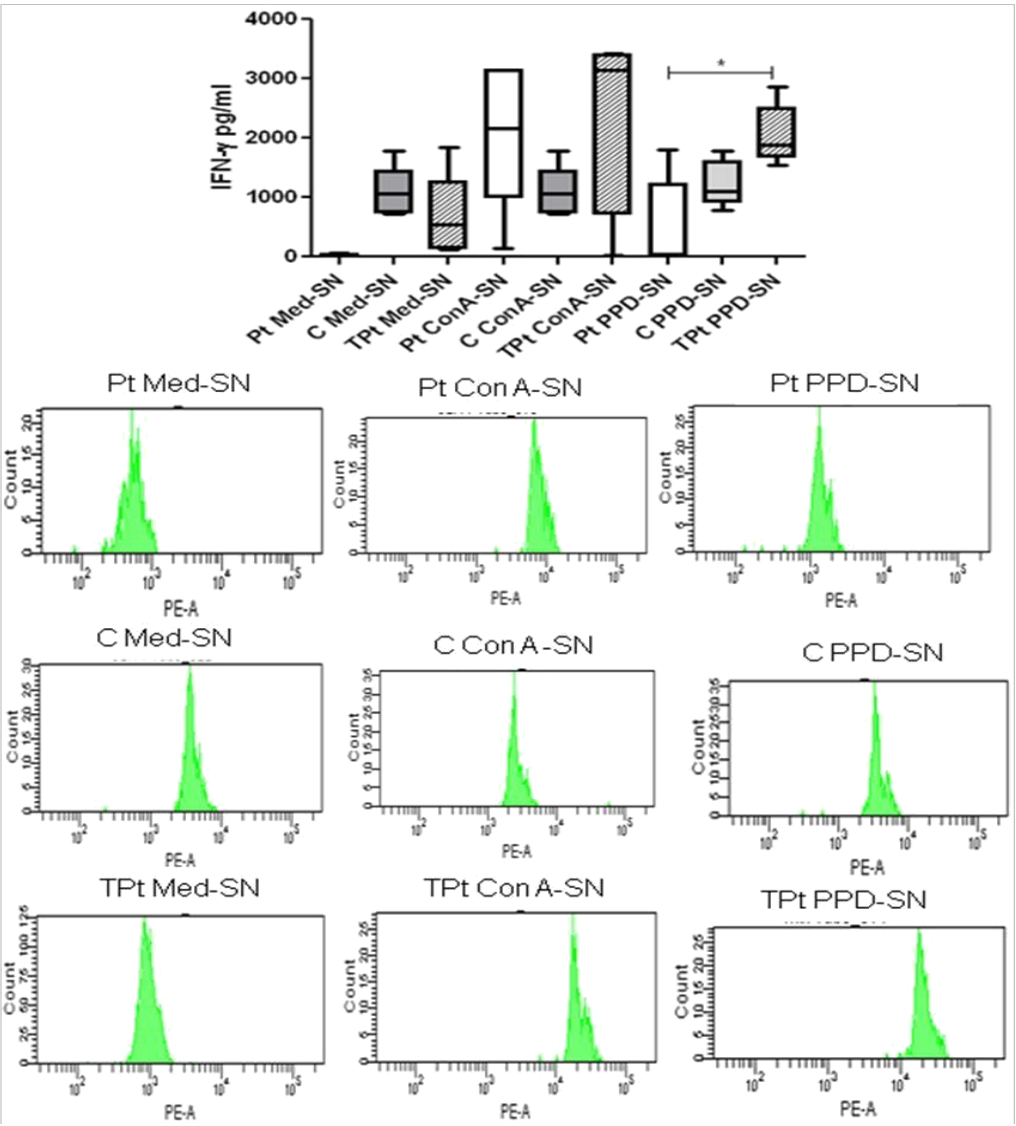


Figure 3.11 Graph and Histograms representing CBA Analysis of IFN-γ in culture supernatant. Culture supernatants obtained from peripheral mononuclear cells of patients before (Pt) and after treatment (TPt) and controls (C) and incubated with ConA (ConA-SN) and PPD (PPD-SN). Control wells were seeded with cells without any antigen/mitogen (Med-SN). The levels of IFN-γ were analysed by cytometric bead array.

Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

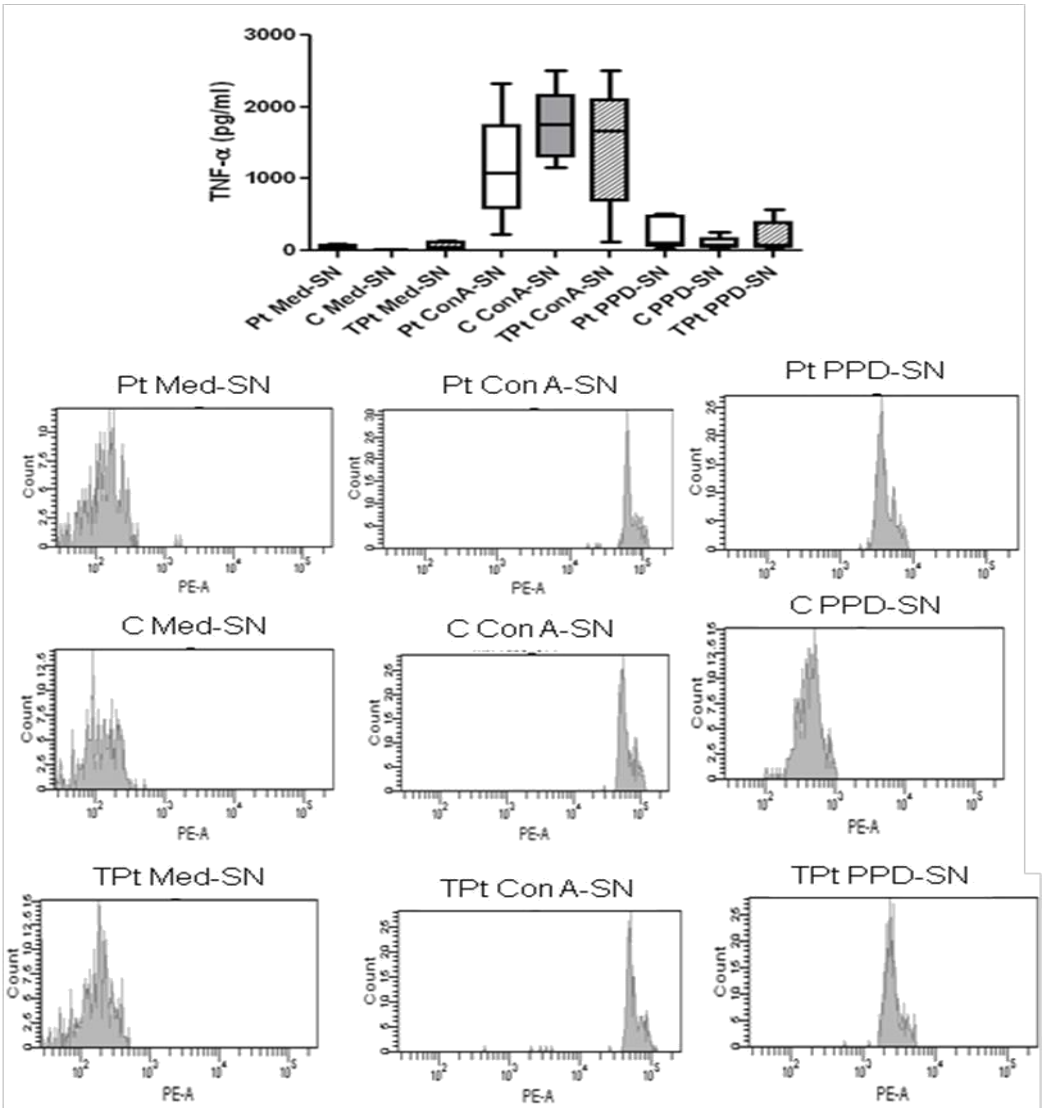


Figure 3.12 Graph and Histograms representing CBA analysis of TNF-α in culture supernatants. Culture supernatants obtained from peripheral mononuclear cells of patients before (Pt) and after treatment (TPt) and controls (C) and incubated with ConA (ConA-SN) and PPD (PPD-SN). Control wells were seeded with cells without any antigen/mitogen (Med-SN). The levels of TNF-α were analysed by cytometric bead array. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni– Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

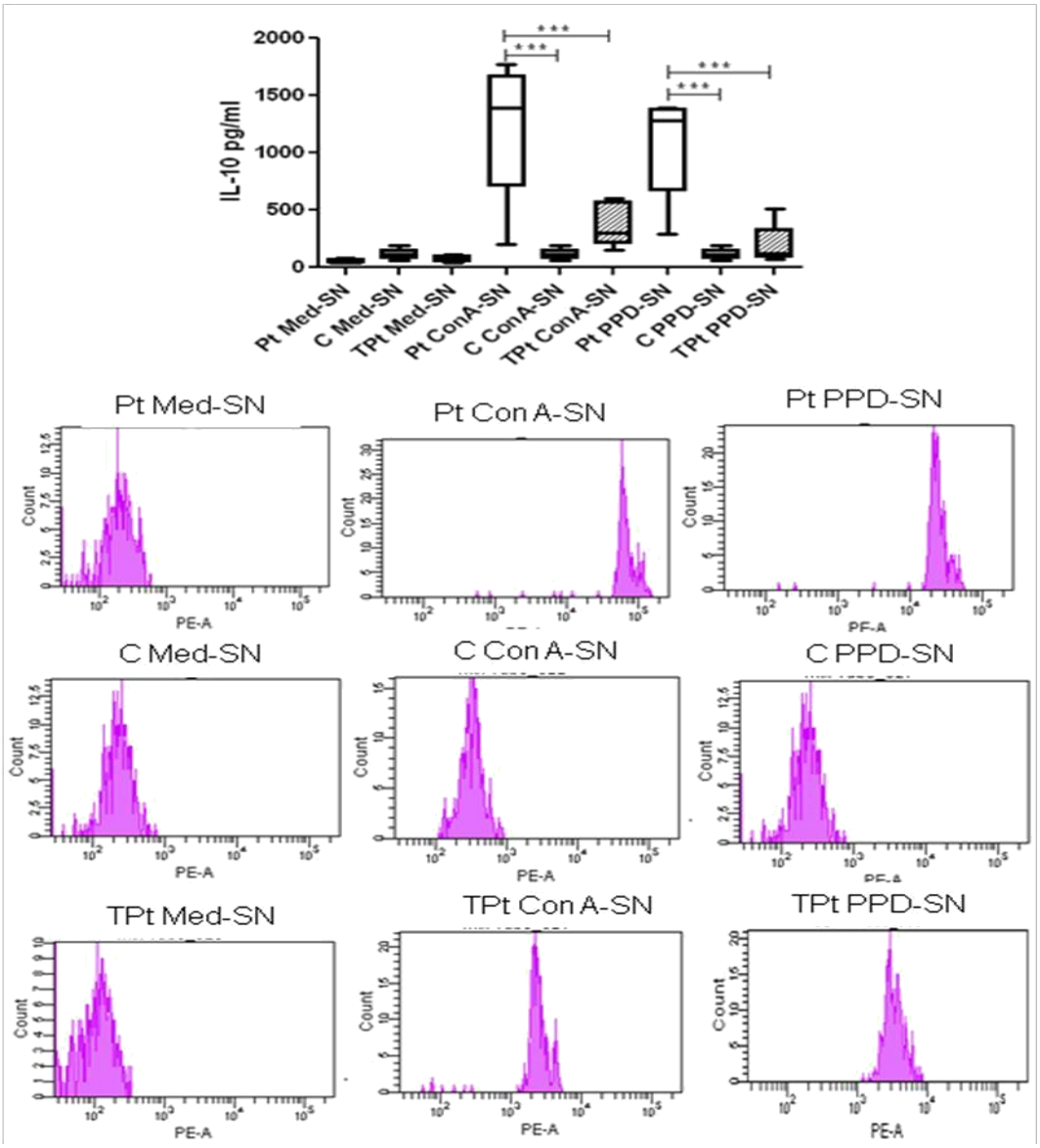


Figure 3.13 Graph and Histograms representing CBA analysis of IL-10 in culture supernatants. Culture supernatants obtained from peripheral mononuclear cells of patients before (Pt) and after treatment (TPt) and controls (C) and incubated with ConA (ConA-SN) and PPD (PPD-SN). Control wells were seeded with cells without any antigen/mitogen (Med-SN). The levels of IL-10 were analysed by cytometric bead array. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni–Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

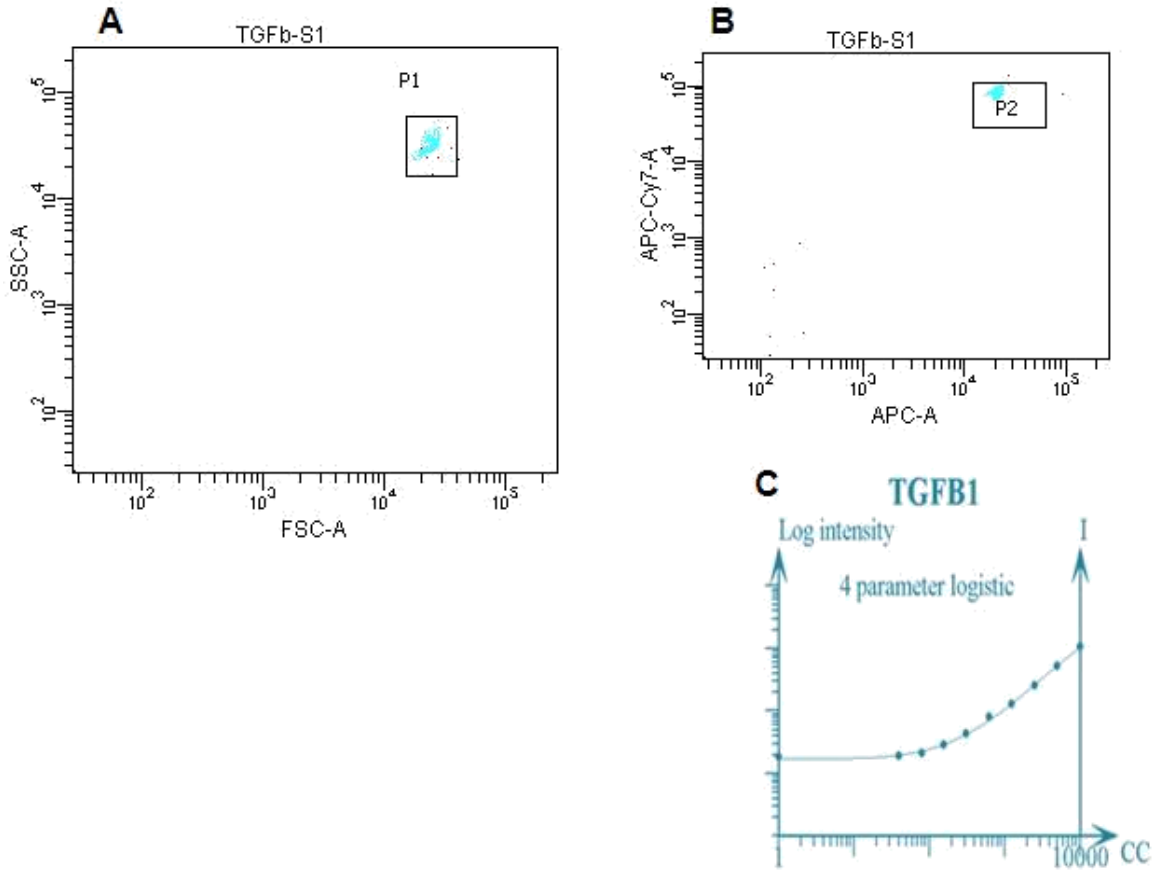


Figure 3.14 FACS plots showing gating and standard graphs of singleplex cytokine bead array. (A) Representative FACS plot showing parental population (P1) of TGF-β cytokine beads (B) Representative FACS plot for TGF-β (P2) population at the B6 position (light Blue). In addition, the representative standard graphs of (C) TGF-β is also shown.

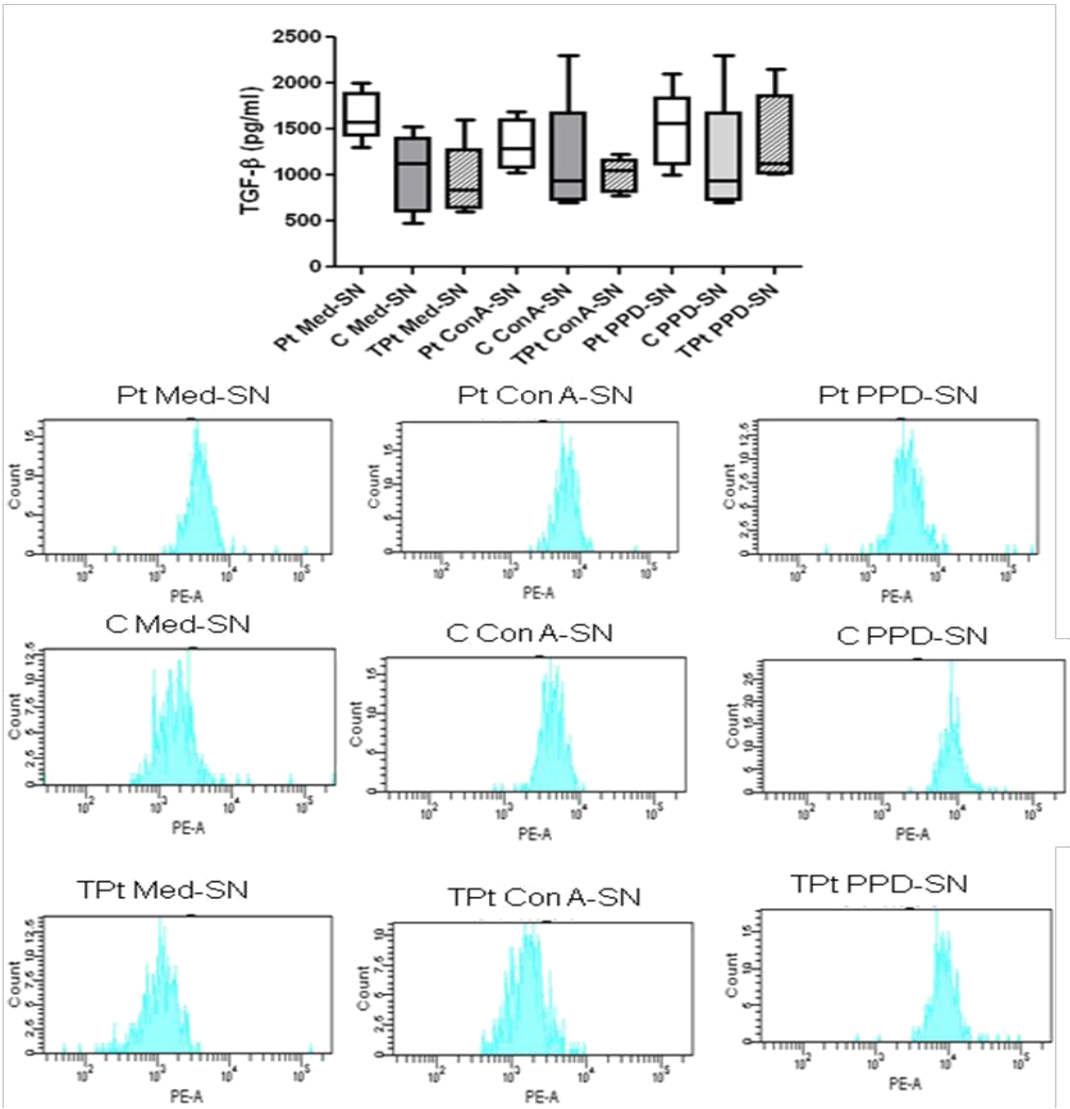


Figure 3.15 Graph and histogram representing CBA analysis of TGF-β in culture supernatants. Culture supernatants obtained from peripheral mononuclear cells of patients before (Pt) and after treatment (TPt) and controls (C) and incubated with ConA (ConA-SN) and PPD (PPD-SN). Control wells were seeded with cells without any antigen/mitogen (Med-SN). The levels of TGF-β were analysed by cytometric bead array. Comparability of groups was analyzed by Mann–Whitney U-test. A Bonferroni– Holm procedure was used to correct for multiple comparisons between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$

Interestingly, it was found that the levels of IL-10 in ConA-SN as well as PPD-SN of patients before treatment were found to be significantly higher than those of patients after treatment ($p<0.001$) and healthy controls ($p<0.001$). This observation further strengthens the findings obtained in the previous experiment that significantly increased IL-10 seems to be overriding the Th1 response (IL-2 and IFN-γ) and thereby exerting its immunosuppressive role leading to decreased MGC formation.

CHAPTER 4

2D-GEL ELECTROPHORETIC ANALYSIS OF MONOCYTES FROM TUBERCULOSIS PATIENTS, HOUSEHOLD CONTACTS AND CONTROLS.

"Research is to see what everybody else has seen,
and to think what nobody else has thought"

Albert Szent-Gyorgyi

CHAPTER 4

2D-GEL ELECTROPHORETIC ANALYSIS OF MONOCYTES FROM TUBERCULOSIS PATIENTS, HOUSEHOLD CONTACTS AND CONTROLS

4.1 INTRODUCTION

Macrophages play an important role in tuberculosis immune response. Following infection, macrophages respond by releasing various cytokines and chemokines that attract different immune cells to the site of infection. These immune cells including neutrophils, lymphocytes, dendritic cells as well as monocytes aggregate to form granuloma, which is the hallmark of tuberculosis. Since monocytes are critical players in the granulomatous response it is essential to examine its role in the immunopathology.

Several studies have been carried out to analyse the changes in monocytes following *M. tuberculosis* infection. In their study using human monocytes, Sanchez et al. (2006) demonstrated a decreased expression of CD14, HLA-DR and CD36 molecules on monocytes infected with *M. tuberculosis*. They also made an interesting observation in that, while monocytes obtained from tuberculosis patients undergo necrosis and apoptosis following infection, the monocytes from healthy controls underwent apoptosis only. Gil et al. (2004) also made a similar observation in their study on monocytes from PPD-positive healthy controls where the monocytes underwent apoptosis when exposed to PPD or *M. tuberculosis,* whereas monocytes of TB patients underwent apoptosis as well as necrosis. Apoptosis of monocytes thereby appears to play a role in the protective immune response, whereas necrosis leads to enhanced tissue damage and facilitates bacterial dissemination. In the same study, it was observed that the phenomena of necrosis could be reverted in tuberculosis patients following anti-TB treatment. Castano et al. (2011) observed the effect of *M. tuberculosis* on the changes in the functionality and differentiation of monocytes to macrophages. Similar to Sanchez et al., they also observed changes in the expression of cell surface molecules in infected monocytes wherein a decreased expression of HLA-II, CD86 and CD36 was observed when

monocytes differentiated into macrophages. Interestingly while on one hand the infection does not affect the ability of bacterial uptake, as observed by the phagocytosis of similar number of latex beads and *M. tuberculosis*-FDA by monocytes, on the other hand, it was observed that these infected monocytes stimulated less proliferation and IFN-γ production in T cells as compared to the uninfected phagocytes which in turn reflects on the low antigen presenting ability of infected cells. The compromised APC function might be partially due to decreased HLA-II and CD86 expression.

As proteins play a crucial role in all biological processes of the cells, the study of the protein levels might shed light on various physiological and pathological processes. The term 'proteome' refers to all measurable proteins in the cells, whereas 'proteomics' refers to the integration of changes in the proteome that reflects the different pathophysiological states. Of late proteomics has been used extensively to identify differences which help in the identification of markers associated with various diseases. Studies have been carried out to determine the differences in proteome during monocyte to macrophage differentiation (Wu et al., 2005) .

Jin et al. (2004) studied the differences in the proteome of monocytes and alveolar macrophages using two-dimensional (2D) electrophoresis/proteomic analysis. They observed that a total of 197 spots were differentially expressed between both the cell types. Out of these, 124 spots showed greater than 2.5 fold increase in protein density whereas less than 2.5 fold change was observed in 73 spots. They identified 35 spots showing higher levels in AMs; they included aldehyde dehydrogenase, Cathepsin B,D , H and X, heat shock protein 27, macrophage capping protein Napsin A and Tripeptidyl peptidase I. Similarly, while studying alveolar macrophage and monocyte proteome, Steinberg et al. (2003) found differences in the levels of approximately 600 phosphorylated proteins. In another study dealing with alveolar macrophage proteome differences between smokers and non-smokers, it was observed that one of the actin derivatives and one isoform of cathepsin D was found only in smokers and not in non-smokers while proteins like galectin-1 and thioredoxin was found in both the groups (Wu et al., 2005).

Several studies have also been carried out for identifying protein signatures in different disease conditions. For example, in case of monocytes obtained from sickle cell anemia patients it was found that transketolase and coronin were most negatively correlated proteins whereas upstream element-binding protein and alpha actinin 1 or alpha actinin 4 were most positively correlated with severity of the disease (Hryniewicz-Jankowska et al., 2009). Likewise in case of monocytes of patients suffering from coronary artery disease there was significant correlation with cytoskeleton protein vimentin and others such as mannose binding lectin receptor and calcium-binding protein S100A8 whereas negative correlation with glutathione transferase and heat shock protein 70 KDa (Poduri et al., 2012).

Although monocytes from tuberculosis patients and controls have been studied as mentioned above, no proteomic study of such monocytes appears to have been done. In this study an attempt has been made to detect any differences in the adherent cell population derived from the peripheral blood mononuclear cells (PBMCs) of patients, House hold contacts (HHC) and healthy controls. The proteomic study was further confirmed by using qPCR and western blot analysis.

4.2 MATERIAL AND METHODS

4.2.1 Isolation of peripheral blood monocytes

Blood monocytes were isolated from peripheral blood of five patients as well as their respective HHC and healthy controls. The patients were identified on the basis of positive sputum acid fast bacilli (AFB) staining and chest X-ray reports. The HHC and healthy controls included in the study are asymptomatic. All patients were males while HHC and controls included both males (two) and females (three) each. The age of the individuals included in the study ranged between 25-40 years of age.

All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S. University of Baroda. Written informed consent was obtained from all subjects.

Isolation of monocytes was carried out within five hours of collection as described elsewhere (Delirezh et al., 2012). 10 mL of blood was collected from each individual in ethylene diamine tetraacetic acid (EDTA) coated vaccutainer tubes and monocytes were isolated by density gradient centrifugation on Ficoll-hypaque followed by adherence, for 2 hours in T25 tissue culture flasks in medium containing RPMI-1640. The non-adhered mononuclear cells were separated from the adhered monocyte preparation by washing vigorously with phosphate buffered saline (PBS). The remaining adherent cells were considered as the monocyte preparation, used in subsequent processing for analysis by 2-dimensional electrophoresis (2-DE).

4.2.2 Protein sample preparation from monocytes

Cell lysis of the monocytes was performed using 1 ml of lysis buffer (5M Urea, 0.25% 3-[(3-cholamidoproplyl) dimethylammonio]-1-propanesulfonate (CHAPS) , 0.25% Tween 20, 100mM Dithio threitol (DTT), 10% isopropanol, 5% glycerol, protease inhibitor, 100mM sodium orthovanadate). This was followed by protein precipitation of monocyte lysate using Methanol Chloroform method (Wessel and Flugge, 1984). Protein

precipitates were then resuspended in 2D sample/ rehydration buffer (Bio-Rad) and the protein was estimated using Bradford assay.

4.2.3 Two-dimensional electrophoresis

Protein concentration for each sample was estimated by Bradford's assay and thereafter 25 µg of protein from each sample was employed per IPG strip (pI 3-10 & pI 3-6). Iso-electric focusing was performed for the protein samples as per the standard protocol with minor changes incorporated to accommodate laboratory conditions. Briefly, the IPG strips were firstly rehydrated at room temperature (RT) over night in 125 µl of rehydration buffer containing 25 µg of protein, in IEF rehydration trays (Bio-Rad Laboratories, USA). At first, strips are rehydrated for 1h at RT and thereafter overlaid with IPG cover fluid (GE Healthcare, USA) and left to hydrate over night. Subsequently the strips are picked up and excess fluid blotted onto a soft blotting sheet, and laid face up into the focusing tray (PROTEAN IEF System, Bio-Rad Laboratories, USA) with the anodic end (+) of the strip resting on the appropriate mark etched on the focusing tray. First dimension isoelectrophoresis (IEF) was performed in three steps: at 250 V for 20 mins, at 4000 V for 2 hrs, 40,000 volt-hours for 4 hours. Before the second dimension, the focused strips were equilibrated in equilibration buffer I for 15 mins at RT and thereafter in equilibration buffer II for 15 mins at RT and thereafter the strip is carefully placed on the resolving gel and sealed with agarose. After SDS-PAGE at 100V, the gel was stained by high sensitivity silver staining kit (Pierce Silver Staining Kit, Thermo Fisher Scientific, USA) as per manufacturer's protocol.

Reagents for 2D gel electrophoreseis

Rehydration Buffer

Urea	8M
CHAPS	2% (w/v)
IPG buffer	0.5% (v/v)
Bromophenol Blue	0.02%
Milli Q water	to 25mL

- Before use 0.5 M DTT is to be added to the stock solution of rehydration buffer.

Bromophenol Blue Stock Solution

Bromophenol Blue	1% (w/v)
Tris-base	50mM
Milli Q water	to 10mL

Lysis Solution

Urea	8M
CHAPS	4%
Biolyte (3-10)	2%
Milli Q water	to 20mL

SDS Equilibration buffer

Tris-HCl, pH 8.8	50mM
Urea	6M
Glycerol (87% v/v)	30% (v/v)
SDS	2% (w/v)
Bromophenol Blue	0.002% (w/v)
Milli Q water	to 200mL

- **Equilibration buffer I contains 200 mM DTT.**
- **Equilibration buffer II contains 250 mM Iodoacetamide.**

4X Resolving gel buffer

Tris base	1.5M
Milli Q water	750mL
HCl	adjust to pH 8.8
Milli Q water	to 1L

Acrylamide gel stock

Acrylamide	30%
bis-Acrylamide	0.8%
Milli Q water	200mL

4.2.4 Spot identification

Spot selected for analysis was excised from silver stained gels and sent to Vimta labs, Hyderabad, India for identification. Briefly, the spots were subjected to in-gel trypsin digestion and peptides generated were subjected to MALDI/TOF analysis. Following the acquisition of peptide mass fingerprint, they were compared with SwissProt data base and the protein was identified.

4.2.5 Total RNA isolation and reverse transcription

To confirm the results obtained from 2D and spot identification, qPCR was done using monocytes of patients (n=10) and controls (n=10). Both the groups included five males and five females each.Total RNA was isolated (as described in chapter 2). The quality of the RNA samples was analyzed by inspecting the integrity of 28S and 18S bands on agarose gel electrophoresis. cDNA was prepared from 1 μg of each total RNA sample using RT-PCR kit (Thermo Fisher Scientific, Surrey, UK). The quality of cDNA preparation was further confirmed by beta actin amplification.

4.2.6 Quantitative PCR

Quantitative PCR (qPCR) was done on a Real-Time thermal cycler (Bio-Rad) using specific primers for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and αII-spectrin. The primers used for αII- spectrin were SPEC FP 5'- AGC AAG CAC CAG AAG CAC CAG G -3' and SPEC RP 5'- TCA GCT AAG GCA GCC AGG CG- 3', and for GAPDH were GAPDH FP 5'- TGA GCA CCA GGT GGT CTC C - 3' and GAPDH RP -5'- TAG CCA AAT TCG TTG TCA TAC CAG - 3'. The qPCR was done according to the following amplification conditions: initial denaturation at 94˚C for 30 s, followed by 45 cycles of denaturation at 94˚C for 10 s, annealing and extension for 30 s at 60˚C and finally the fluorescence was recorded. The amplification was done in triplicate for each sample and cycle threshold (Ct) was used for gene expression analysis. The expression level of αII-spectrin in each sample was normalized to GAPDH expression of the same sample with the help of CFX manager software (Bio-Rad) for gene expression

analysis. The product specificity was confirmed by single peak in melt curve analysis. To check the genomic DNA contamination in sample, the negative controls were set with the total RNA without reverse transcription which did not give any recordable fluorescence (data not provided). Statistical analysis of the data was done using unpaired Students t test ($p<0.05$).

4.2.7 Western Blot Analysis

The proteins separated after SDS-PAGE were transferred to nitrocellulose membranes. After blocking for 1 hour in PBS containing 1% gelatine the membrane was incubated with mouse anti-αII-spectrin monoclonal antibody (1:1000, BD Bioscience) overnight at 4°C. The membrane was then washed thrice with PBS containing 1% Tween-20 followed by 1 hour incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. Subsequently, the membrane was washed thrice with PBS containing 1% Tween-20 and immunoreactive bands were detected by using TMB/ H_2O_2 (Bangalore, Genei).

4.2.8 Monocyte adhesion assay

Peripheral blood mononuclear cells (PBMC) were plated at 10^5/ml per well in 24 well plate for 2 hours. Eight wells were seeded for each sample. Following 2 hours of incubation, four wells were washed with RPMI 1640 to remove unadhered cells. Out of the four, RPMI 1640 media was replaced in two wells and in another two wells RPMI 1640 was added along with PPD (10µg/ml). Unadhered cells were not removed from other set of four wells except for addition of PPD (10µg/ml) in two wells. The plate was incubated overnight at 37°C, 5% CO_2. All the wells were then washed twice with RPMI 1640 and fixed with 4% paraformaldehyde followed by staining with 3 mM calcein AM (Sigma-Aldrich, India) for 30 minutes. Calcein AM was removed, PBS was added to the wells and the wells were then analysed by fluorescence microscopy. Five images were acquired for each sample and the flouresencent cells were counted using ImageJ software. The difference in significance between patient and control samples was statistically analysed using unpaired students t test. ($p<0.001$)

4.3 RESULTS

4.3.1 Differential protein expression in tuberculosis patients

It was hypothesized that monocytes from tuberculosis patients, their HHC and healthy controls would have distinct proteome profiles. The total protein content of monocytes obtained from five individuals of each group was therefore analysed. Since the focus of this study was to identify a few proteins that are either positively or negatively correlated with disease state, a small amount of the protein extract was analysed on a 7 cm IPG strip followed by analysis on 7x8 cm SDS-PAGE gels. Using PD Quest software, approximately 450-650 spots could be detected in these gels as can be seen from the figure (Fig.4.1 and 4.2) which has representative 2D gel of patients (Fig. 4.2A & 4.2B), HHC (Fig. 4.2C & 4.2D), and healthy controls (Fig. 4.2E & 4.2F), following 2D separation of 25µg of protein sample, and silver staining. It is apparent that this is a very small fraction of the protein content of monocyte when compared to that reported by others (Jin et al., 2006). Since the focus of the present investigation was to detect differences between the proteome of monocytes obtained from tuberculosis patients, their HHC and healthy controls, loading of a greater amount of protein on the strips used would make resolution of individual spots more difficult. As can be seen from the figure (Fig. 4.2), which are the magnified images of selected area of the above mentioned 2D gels of patients (Fig. 4.2A & 4.2D), HHC (Fig. 4.2B & 4.2E) and controls (Fig 4.2C & 4.2F) respectively, only a small number of proteins are visibly up or down regulated under these conditions.

4.3.2 Protein identification

Following MALDI/ TOF analysis, one of the spots (spot 1) was identified as α II-spectrin which was found to be absent from monocytes of all patients, and not that of any of the HHC or healthy controls. Protein identification following acquisition of peptide mass fingerprint was based on 14 mass values being matched out of a total of 30 mass values searched, and covering 7% of the sequence (Fig. 4.3). Also, the identified protein had a Mowse score of 55, where a Mowse score of 53 at $p<0.05$ was considered

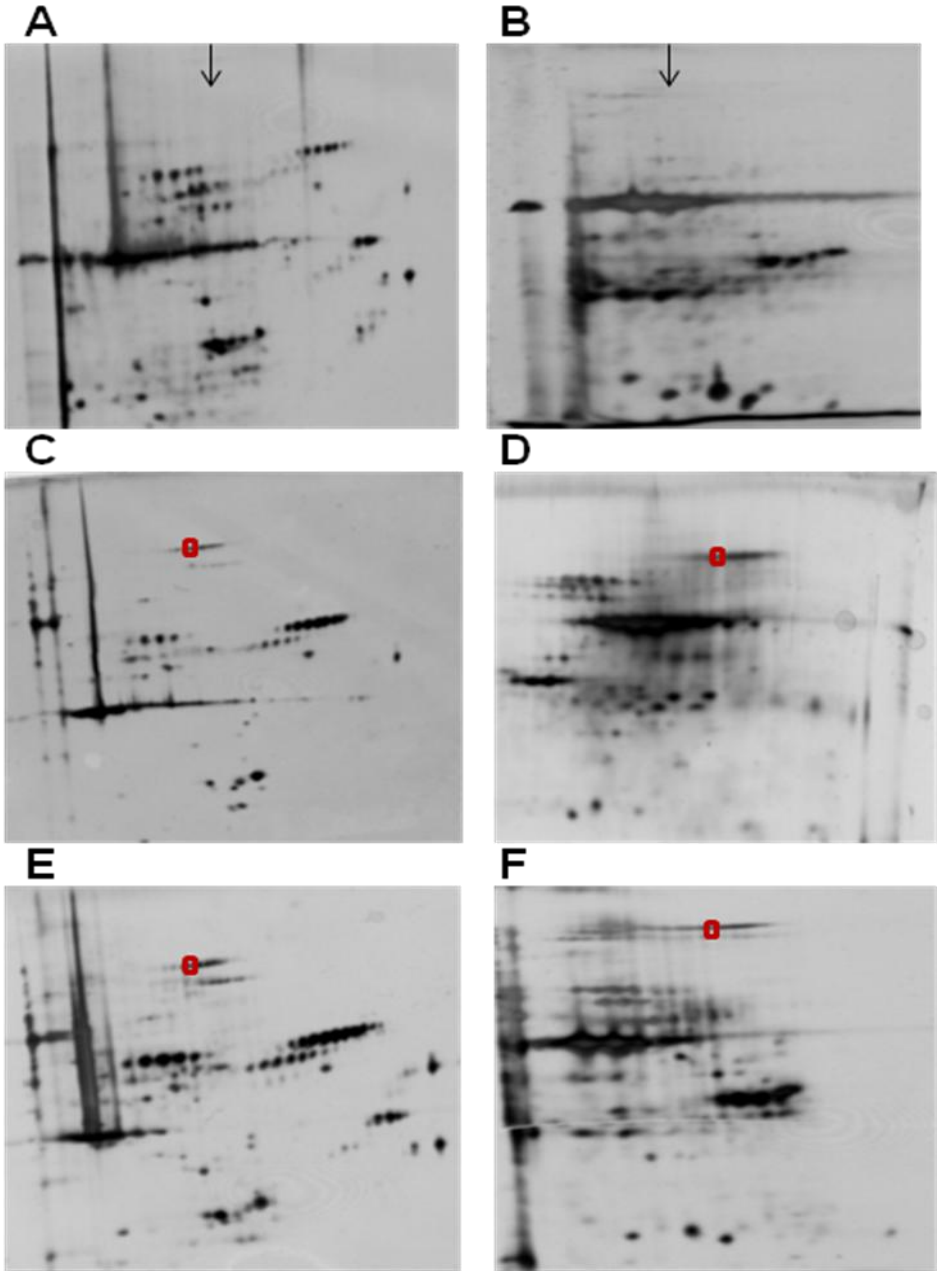


Figure 4.1 2D protome maps of monocyte total protein. Silver stained 2D gel electrophoresis of protein obtained by monoyctes of two active tuberculosis patients (A, B), two HHC (C,D) and two healthy controls (E,F) was done using Immobiline dry strips (IPG) of narrow range (3-6L). The highlighted spot show the absence (A,B) and presence (C-F) protein spot which was later identified as αII-spectrin.

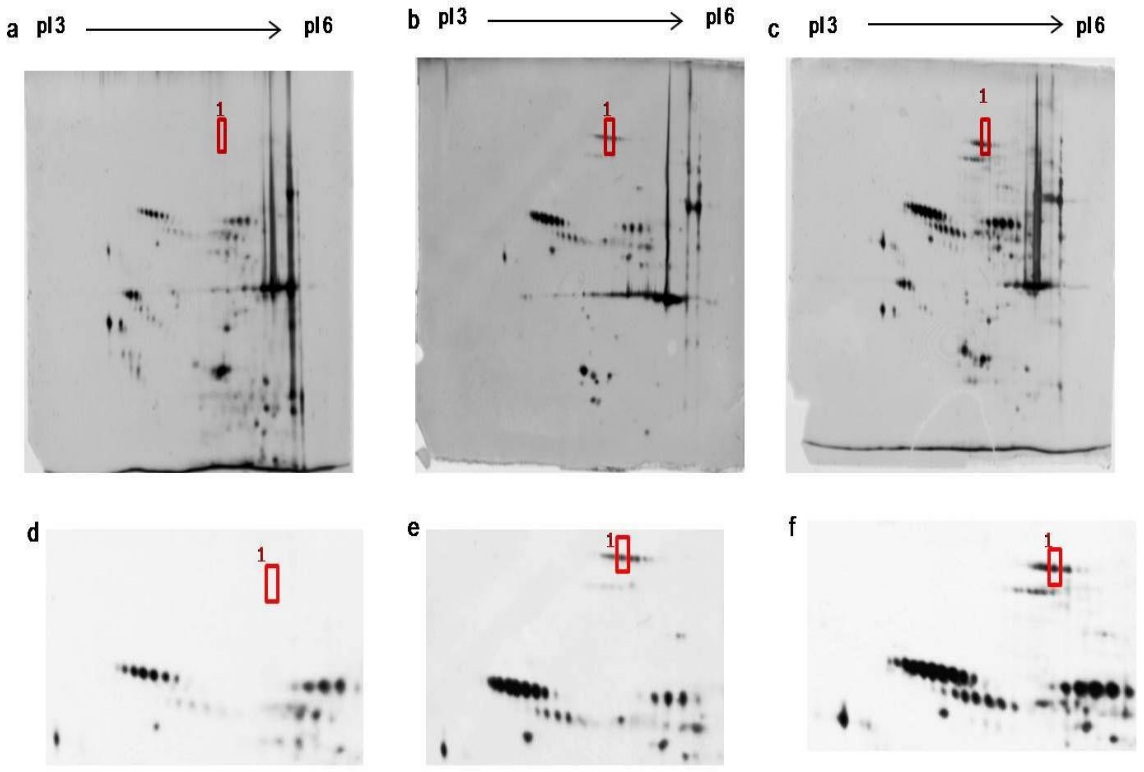


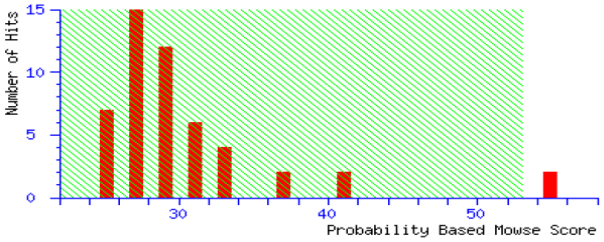
Figure 4.2. Representative 2D proteome maps of monocyte total protein. Siver stained 2D gel electrophoresis of a) active tuberculosis patient, b) corresponding household contact and c) healthy controls using IPG strips of narrow range (3-6L). Magnified images of selected areas of the above 2D gels show the presence or absence of the highlighted spots in d, e and f respectively. Protein spot 1 was identified as αII-spectrin

Mascot Search Results

```
User            :
Email           :
Search title    : SampleSetID: 461, AnalysisID: 878, MaldiWellID: 25419, SpectrumID: 8502, Path=\201
Database        : SwissProt 20050315 (176469 sequences; 63878124 residues)
Taxonomy        : Homo sapiens (human) (12019 sequences)
Timestamp       : 24 May 2012 at 11:54:53 GMT
Top Score       : 55 for SPTA2_HUMAN, (Q13813) Spectrin alpha chain, brain (Spectrin, non-erythroid
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 53 are significant (p<0.05).



Protein Summary Report

Format As | Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 10

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	SPTA2_HUMAN	285150	55	(Q13813) Spectrin alpha chain, brain (Spectrin, non-erythroid alpha

1. SPTA2_HUMAN **Mass:** 285150 **Score:** 55 **Expect:** 0.04 **Queries matched:** 14
(Q13813) Spectrin alpha chain, brain (Spectrin, non-erythroid alpha chain) (Alpha-II spectrin

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
947.5596	946.5523	946.4906	0.0618	982	989	1	K.SPREVTMK.K
1138.6074	1137.6001	1137.6254	-0.0253	2048	2057	1	K.HVQSKAIEAR.H
1475.7948	1474.7875	1474.7086	0.0790	1926	1938	0	R.VNDVCTNGQDLIK.K
1495.8418	1494.8345	1494.7361	0.0984	2304	2315	0	R.MQHNLEQQIQAR.N
1527.8468	1526.8395	1526.8245	0.0150	1845	1856	1	R.LAQFVEHWKELK.Q
1701.9469	1700.9396	1700.8005	0.1391	1487	1500	1	K.HEDFDKAINVQEEK.I
1707.7969	1706.7896	1706.8528	-0.0632	567	580	0	R.AQLADSFHLQQFFR.D
1761.8921	1760.8848	1760.8805	0.0043	1699	1713	1	K.KHQLLEADISAHEDR.L
1900.0032	1898.9959	1898.8654	0.1305	2383	2398	0	R.DGHVSLQEYMAFMISR.E + Oxidation (M
1940.9679	1939.9606	1939.9904	-0.0298	1839	1853	1	K.EEIQQRLAQFVEHWK.E
1972.9890	1971.9817	1972.0186	-0.0369	2264	2281	1	K.KIEDLGAAMEEALILDNK.Y
2034.0874	2033.0801	2033.0065	0.0736	64	81	1	K.LQIASDENYKDPTNLQGK.L
2147.0874	2146.0801	2145.9926	0.0875	419	439	0	K.SADESGQALLAAGHYASDEVR.E
2256.0496	2255.0423	2255.0099	0.0325	2436	2454	1	R.EQADYCVSHMKPYVDGKGR.E + Oxidation

No match to: 870.5417, 1124.5634, 1156.6057, 1362.5760, 1377.7336, 1382.6895, 1526.8594, 1546.8087, 1612.7897, 1710.8203, 1830.0123, 1898.9985, 1913.0161, 1914.0198, 2163.0652, 2634.4446

Figure 4.3. Result of the MALDI/TOF analysis. Peptide mass fingerprinting results show 14 mass values being matched to αII-spectrin out of 30 mass values covering 7% of sequence. The identified protein had Mowse score 55. Mowse score of 53 is considered significant at p<0.05.

significant (Fig. 4.3). Although there are other differences, we are currently unable to establish the identity and significance of these.

4.3.3 qPCR analysis of αII-spectrin

As mentioned the objective of this study was also to confirm this finding by quantitative PCR to see if it could be used to differentiate these two groups. Separate samples of ten individuals each from the patients and controls was obtained for monocyte isolation. As can be seen from the graph, αII-spectrin level normalized to GAPDH is significantly greater ($p=0.0002$) by approximately 15 to 55 folds in controls when compared to patients (Fig. 4.4A).

4.3.4 Western Blot Analysis

In order to confirm the levels of α II-spectrin, monocyte preparations were also subjected to western blot analyses, using anti- α II-spectrin monoclonal antibody as a probe. As seen in Fig. 4.4F bands corresponding to approximate molecular weight 120 and 150 KDa can be seen only in samples derived from control monocyte lysate (Fig. 4.4F lanes 4,5,6) and not patients monocytes (Fig. 4.4F lanes 1,2,3).

4.3.5 Monocyte adhesion assay

In order to analyse the adhesion property of monocytes in tuberculosis patients in comparision to that of healthy controls monocyte an adhesion assay was performed. The adhered PBMC were stained with calcein-AM dye and enumerated under fluorescence microscope. As shown in Fig. 4.5 there was a significant decrease (2 fold) in adherent population of patients as compared to healthy controls.

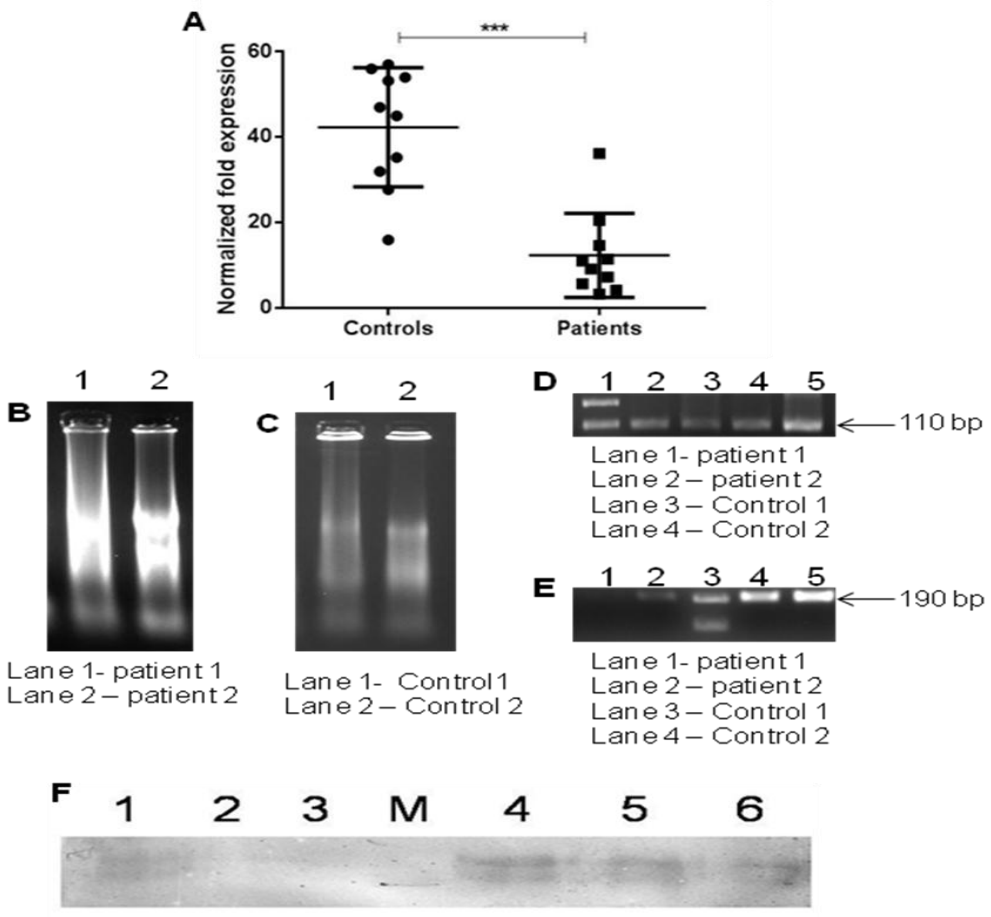


Figure 4.4 Quantitative PCR and Western blot analysis of αII-spectrin gene. A) Graph showing fold expression of αII-spectrin gene in monocytes of tuberculosis patients and controls which was normalized against GAPDH gene expression. Monocytes from patients (n=10) and controls (n=10) were studied. Each sample was analysed in triplicate. Error bars indicate standard deviation. Statistical analysis was done using unpaired students t test. ($p<0.05$). Representative gel picture of 0.8% agarose gel with ethidium bromide stained samples of RNA samples of patients (B) and healthy controls (C) and amplicons obtained from qPCR which was done using specific primers for GAPDH (D) and αII-spectrin gene (E) of both patients and controls. F) Western blots using anti-αII spectrin monoclonal antibody showing bands of 120 Kda and 150Kda - lanes with patient samples (lanes : 1,2,3) and control samples (lane: 4, 5, 6).

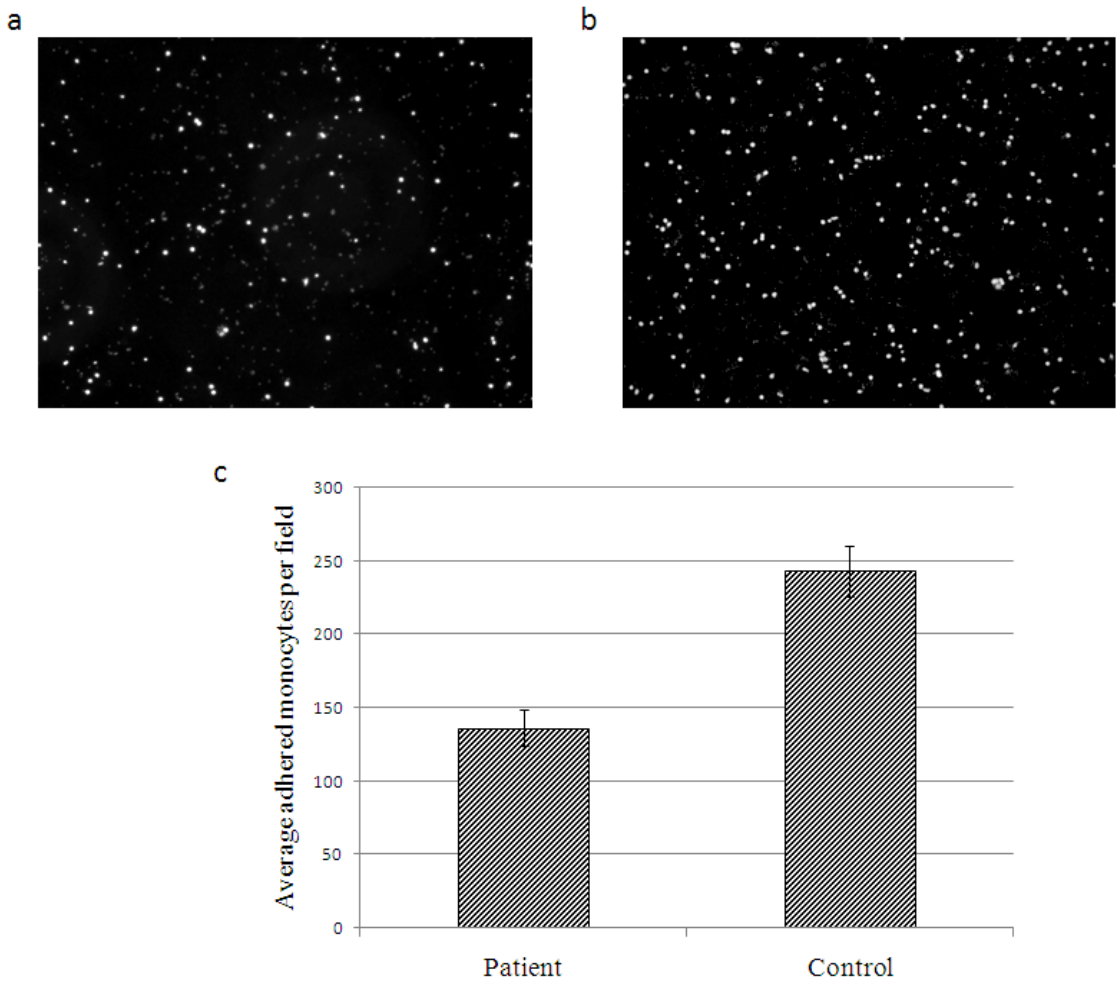


Figure 4.5 Monocyte adhesion assay using calcein-AM dye. Representative fluorescent microscopy pictures showing calcein stained adhered monocytes of patients (a) and controls (b) and graph showing average number of adhered monocytes per field in patients and controls (c).

4.4 DISCUSSION

Tuberculous granuloma consists of infected macrophages surrounded by many cells like fibroblasts, leukocytes and monocytes recruited from the periphery. The significance of the newly recruited monocytes that finally forms the granuloma is still undefined. Recent studies have shown the altered ability of *M. tuberculosis* infected monocytes isolated form healthy individuals to differentiate into macrophages (Castano et al., 2011). The objective of this study was therefore to detect differences in the monocyte proteomes of patients, HHC and healthy controls. Results obtained demonstrate α II-spectrin as a major difference between the proteomes. Alpha II-spectrin has been found to be present in HHC and controls but not in patients (Fig 4.2). This has been substantiated, with the help of qPCR, taking a housekeeping protein GAPDH as a control (Fig 4.4).

Spectrins are large flexible proteins that have a scaffolding function being involved in the organisation and stabilisation of membrane microdomains. They consist of α- and β-subunit isoforms. In addition to their association with the actin network, they are also known to be associated with various proteins involved in diverse functions (Bennet and Healy, 2008; Moon and McMohan, 1990). It has been recently shown using siRNA knock out cell line that deficiency of the non-erythrocytic isoform α II-spectrin identified in the present study, results in adhesion defects which could lead to cell cycle arrest. In the same study, it was observed that the eventual cell cycle arrest was mediated by defects in actin reorganisation and integrin distribution (Metral et al., 2009).

In other studies it was also demonstrated that spectrin cytoskeleton has a significant role in adhesion and invasion of enteropathogenic bacteria (Ruetz et al, 1994). It is therefore conceivable that a down regulation in the expression of α II-spectrin could compromise the functionality of blood monocytes and therefore be a mechanism by which *M. tuberculosis* establishes an eventual diseased state. While the present study has used the adherence ability of monocytes to separate them from peripheral blood, this does not conflict with the findings reported by Metral et al. (2009) where also, siRNA mediated knock out of α II-spectrin has not resulted in total ablation of cell adhesion.

Furthermore, it is possible that it has similarly impacted the patients' monocytes included in this study; this aspect of reduced adhesion was investigated by using a monocyte adhesion assay. On comparing the adherent cell population of both patients and controls by calcein staining, it was observed that the average number of adhered monocytes per field in case of patients' monocytes were significantly less than that of healthy controls' monocytes. This implies that the compromised adhesion ability of patient monocytes in comparison to control monocytes appears to be due to decreased α II-spectrin expression.

The results (Fig.4.4) demonstrate that lysates obtained from controls contain the breakdown products of αII-spectrin while they are absent in case of patients. The function of spectrin being maintenance of cell shape and structure, it is surprising to see breakdown products of α II-spectrin in the control samples. This however could be a consequence of *in vitro* differentiation of monocytes to macrophages as evidenced by other studies (Bocchino et al., 2005, Ciaramella et al., 2000, Zhang et al., 2009). It is seen in those studies that monocytes differentiate to macrophages when incubated in media containing serum (Bocchino et al., 2005), as well as that caspase 3 which is activated during differentiation (Ciaramella et al., 2000) is responsible for cleavage of α II-spectrin to give 120 KDa and 150 KDa breakdown products (Zhang et al., 2009). Based on these observations, the reason we do not see either intact (in 2D gels) or breakdown products (in both 2D and western blots) of α II-spectrin in patients is probably because spectrin levels in general are reduced in patients as substantiated by qPCR, 2D and western blot data and thus are defective in *in vitro* differentiation to macrophages.

The contention that the monocyte functionality is affected by lower levels of αII-spectrin, is substantiated by the observation that monocytes from tuberculosis patients are severely affected in their functions and have also been shown to undergo apoptosis during differentiation to macrophages (Ciaramella et al., 2000, Zhang et al., 2009). In the present study, monocyte proteome of tuberculosis patients, HHC and controls revealed that αII-spectrin to be one of the spots downregulated in patients. The decreased expression of αII-spectrin in tuberculosis patients was further confirmed by qPCR and western blot

analysis. Therefore, this study suggests the possible role of decreased levels of αII spectrin in the pathology of tuberculosis.

CHAPTER 5

STUDY OF CYTOTOXIC T LYMPHOCYTES IN PATIENTS AND CONTROLS

"Our knowledge can only be finite,
while our ignorance must necessarily be infinite"

Sir Karl Popper

CHAPTER 5

STUDY OF CYTOTOXIC T LYMPHOCYTES IN PATIENTS AND CONTROLS

5.1 INTRODUCTION

The traditional approaches of using BCG and PPD for vaccination and diagnostics respectively have not yielded the desired effect of controlling the disease. Therefore, over the years, a lot of effort has gone into the development of better diagnostics and candidate vaccines, for the control of this pathogen. Being an intracellular pathogen, it is generally believed that protection against this organism would require a coordinated cell mediated response.

Consequently, studies have shown that T cells play a crucial role in developing resistance to disease following Mtb infection (Flynn and Ernst, 2000). In the mouse model, it is known that within one week of infection, increasing numbers of both CD4 and CD8 T cells appear in the draining lymph nodes (Feng et al., 1999). Furthermore, within 3-4 weeks post infection, they migrate to the lungs (Mustafa et al., 2009) and in both humans and mice, are also found to populate the tuberculous granulomas (Kipnis et al., 2005). The earliest evidence indicating a role for T cells highlighted the importance of CD4 T cells in immunity to TB. The importance of CD4 T cells in immunity to TB has been extensively studied in both the mouse and human models. CD4 T cells from infected mice and humans are known to produce IFN-gamma, to a variety of mycobacterial antigens, in vitro (Deenadhayalan et al., 2001; Lalvani et al., 1998). IFN-gamma has an important role in the control of Mtb infection. IFN-gamma knockout mice (Lalvani, 2002), as well as individuals defective in IFN-gamma or IFN-gamma receptor genes (Lalvani et al., 1998) are highly susceptible to Mtb infection. Finally, mice depleted of CD4 T cells were shown to succumb to even low doses of Mtb exposure (Scanga et al., 2008).

Of late there has been increased attention paid to the CD8 subset. Studies conducted in different mice models have demonstrated that CD8 T cells also have a role to play in controlling *M. tuberculosis* infection. Mice lacking β2 microglobulin or TAP1 and therefore depleted of CD8 T cells, showed reduced control of infection when compared to control mice (Behar et al, 1999; Rolph et al., 2001). However it appears that although CD8 cells may be induced early during infection, they play a significant role only in the later phases of infection. Mycobacterial antigens recognised by CD8 T cells in both humans and mice have also been identified. Furthermore, several CD8 specific epitopes have been identified in antigens such as ESAT-6, CFP10, Ag85A, and Ag85B.

While the role of CD8 T cells in protection against murine tuberculosis is well established (Flynn et al, 1993; Orme et al., 1993; Rolph et al., 2001), in the case of humans its importance is being realized only now (Behr et al., 1999, Hussain et al., 2007). This could be due to the difficulty in identifying and isolating Mtb specific class I restricted CTLs from infected hosts, either due to technical reasons or due to the low frequency of specific CTLs. Nevertheless, since the initial reports about the presence of class I restricted CTLs in MTB infected humans, several lines of evidence implicate these T cells in immunity to human TB. Using a novel and sensitive approach for detecting human CD8 T cells for the first time, Lalvani et.al. (1998) were able to establish a role for ESAT-6 and Ag85 complex specific CTLs in host defense against Mtb. Both these antigens are secreted proteins of Mtb and have also been implicated in animal protection studies (Al- Attiyah et al., 2008; Serbina et al., 1999).

While the above human study demonstrated CTL activity against endogenously expressed Mtb secreted antigens, it lacked evidence for cytolytic activity against infected macrophages. Using a different approach, Cho et. al. (2000) screened Mtb protein antigens for peptides with affinity for a particular HLA class I molecule and used an HLA transfected target cell line. Using these, they demonstrated the existence of Mtb peptide specific CTLs. These CTLs were further demonstrated to have protective function as exhibited by their in vitro peptide specific IFN-gamma release, as well as the ability to lyse Mtb infected THP-1 macrophages. The CTLs used in the study were

however, generated by pulsing homologous dendritic cells and PBMCs (which could also give rise to dendritic cells), with respective peptides. A slightly modified approach was used for generating CTLs specific for a non-secreting antigen, Mbt39 in a different study (Kong et al., 2005). This study used recombinant adenovirus infected homologous dendritic cells as APCs in generating CTLs in vitro. The CTL clones in this study too were demonstrated to release IFN-gamma, in an antigen specific way, as well as lysed Mtb infected macrophages.

The observation that live vaccines could provide better protective immunity, led to the assumption that protective T cells could recognise antigens secreted by the organism during growth. Several studies indicated the presence of CTLs from infected animals as well as humans (Raja, 2004, Serbina and Flynn, 1999), that were specific for such secreted antigens. Therefore, studies were conducted to evaluate the possibility of using Mtb secreted antigens as protective antigens. Consequently, it was shown by several workers that immunization with secreted antigens of Mtb conferred protection in mice and guinea pigs (Baldwin et al., 1998). In the above studies with human CTLs too, the secreted antigen ESAT-6 and Ag 85 complex have been examined and found to be responsible for antigen specific cytolysis of Mtb infected macrophages.

Vaccination employing BCG results in the induction of both CD4 and CD8 responses, considered to be important in protection against tuberculosis (Mustafa and Al-attiyah,2009; Skjot et al., 2002, Vekemans et al., 2004). Although these responses are directed against antigens expressed by both the virulent (H37Rv) and avirulent (H37Ra) forms of *M. tuberculosis*, BCG vaccination does not guarantee protection against MTB. The question therefore arises as to whether there are other more important antigens that are not provided by the BCG vaccine or, on the other hand it is also possible that antigens expressed by virulent *M. tuberculosis* but not BCG, induces a non-protective immune response. Therefore it becomes important to study the cytokine response of tuberculosis patients, in response to antigens encoded by the region that are present or absent from various Mycobacterial genomes. Furthermore, as mentioned above, due to the

inefficiency of the widely used BCG vaccine, it is pertinent to study those regions that are present in virulent MTB, but absent in the BCG vaccine.

Comparative genomic studies have led to large scale sequence comparisons among various Mycobacteria. Analysis of various Mycobacterial genomes using different techniques such as subtractive hybridisation, bacterial artificial chromosome libraries and DNA microarrays have led to the identification of several regions that are different among members of Mycobacteria. Using subtractive hybridisation, initial studies comparing the genomes of virulent *M. tuberculosis* and *M. bovis*, and vaccine strain BCG indicated the absence of 3 regions (RD1 to RD3) from the genome of BCG, compared to the other two (Mahairas et al., 1996). However, subsequent studies using more advanced techniques revealed the absence of another 13 RDs (RD4- RD16) from the genome of *M. bovis* compared to *M. tuberculosis* (Behr et al., 1999, Flynn et al., 1993). Studies have been carried out in order to identify and evaluate specific antigens from these regions, for the development of better diagnostics and more effective vaccines (Al-Attiyah and Mustafa, 2008; Mustafa et al., 2008).

Following the sequencing of the genome, *M. tuberculosis* has been shown to harbour four phospholipase C (Plc) genes. While three of them are clustered together in the region designated as RD5, the fourth is located in a different genomic region called RvD2. Phospholipase C plays an important role in the pathogenesis of a number of bacterial infectious agents including *Pseudomonas aeruginosa*, *Bacillus cereus*, *Clostridium perfringens*, and *Listeria monocytogenes* (McNamara *et al*., 1994). In the case of *L. monocytogenes* which is a Gram positive intracellular pathogen, there are two phospholipase C enzymes. Inactivation of the phosphatidyl inositol specific Plc A results in phagosome lysis. This eventually results in preventing replication within mouse peritoneal macrophages and therefore propagation in the host tissue (Camilli et al., 1991). The mode of action of the other phospholipases in the development of pathogenesis is however different. The haemolytic phospholipase C of *P. aeruginosa* is known to suppress neutrophil respiratory burst by interfering with a protein kinase C specific signalling pathway (Terada et al., 1999). On the other hand, the alpha-toxin of *C.*

perfringens has a more systemic effect and has demonstrated dermonecrotic, vascular permeabilising and platelet aggregating properties (Titball, 1993)

Owing to the established role in the pathogenesis of the above mentioned bacteria, Plcs have also been studied in Mycobacteria. Several genetic and biochemical studies have indicated a role for the phospholipases in the pathogenicity of the organism (Raynaud et al., 2002, Talarico et al., 2005). The RD5 deletion which is absent from *M. bovis* and *M. bovis* BCG, but present in *M. tuberculosis* H37Rv and H37Ra includes three genes that encode phospholipase C enzymes. These are *plcA* (Rv2351c), *plcB* (Rv2350c), and *plcC* (Rv2349c). Although these genes are absent in *M. bovis*, a very low level of Plc activity has been reported in *M. bovis* but not in *M. bovis* BCG. This has been attributed to another gene *plcD*, located in a different region RvD2, which is responsible for the residual Plc activity (Flynn et al., 2004). Comparing the plc activity of *M. bovis* and *M. tuberculosis*, it was seen that while it is absent in BCG strains, it was lower in *M. bovis* compared to *M. tuberculosis* (Johansen et al., 1996, Raynaud et al., 2002, Wheeler and Ratledge, 1992). These observations are in agreement with the above mentioned genetic studies which indicate that plc genes are absent from BCG but present in *M. tuberculosis* and barely represented in *M. bovis*, in the form of the *plcD* gene. Using DNA microarray analysis in a different study, (Rehren et al., 2007) it was shown that *plcB* and *plcC* were expressed almost 70 fold higher in MTB H37Rv compared to *M. bovis*. The fold increase observed for *plcA* was however only 19. These observations establish the importance of phospholipase C in the growth of the bacteria.

In a detailed analysis of the contribution of phospholipases in *M. tuberculosis* pathogenesis, mutants inactivated in each of the *plc* genes, as well as multiple *plc* genes were studied (Raynaud et al., 2002). It was concluded from this study that all the *plc* gene products were equally important for the growth of the organism. However, while the mutants did not shown any attenuation in their growth within human monocytic THP-1 cells, they did exhibit an attenuated proliferative capacity in the lungs and spleen of infected mice. This observation, along with the fact that these genes showed a temporal

pattern of expression, indicated their importance as virulence factors in persistent infection.

Studies that have been conducted for looking at peptide specific immune response in tuberculosis are mostly restricted to antigens such as ESAT-6, CFP-10, PPE proteins, MBP70, Ag85 A, -B, and –C and practically none involving the phospholipases. In one such study as mentioned earlier, peptide pools corresponding to the RD region where the phospholipases are represented were used. Overlapping peptides were synthesized covering the entire ORFs specified in various RD regions (Mustafa et al., 2009). It was seen from this study that the peptide pool corresponding to this region where the phospholipases are represented, showed a significant IFN-γ response when PBMCs from both tuberculosis patients and controls were stimulated. This IFN-γ response however, was not as strong as seen with peptide pool corresponding to the RD1 region which encompasses ESAT-6, CFP-10, besides others. It was also shown in the same study that peptide pool corresponding to the RD9 region, could induce a significant IL-10 response. Furthermore, in peptide mixing experiments of the same study it was observed that RD9 specific peptide pool was capable of dampening the strong IFN-γ response induced by RD1 specific peptide pool.

The importance of the phospholipases in the physiology of *M. tuberculosis* is brought out by another study which demonstrated that these genes were specifically up regulated in *M. tuberculosis*, when compared to *M. bovis*, under in vitro culture conditions (Rehren et al., 2007). It can therefore be concluded that besides other antigens, the phospholipases have the potential to induce a strong and protective IFN response, which is not achieved in BCG vaccinees. This study therefore intends to analyse the immune response to synthetic peptides derived from the phospholipases. Specifically, cytokine profiles (IFNγ, TNFα, IL-10, TGFβ) of PBMCs from tuberculosis patients, contacts, and controls, induced by peptides derived from these proteins will be studied.

5.2 MATERIALS AND METHOD

5.2.1 *In silico* analysis of *M. tuberculosis* antigens with protective and susceptible HLA alleles

The antigens and HLA alleles included in *in silico* analysis were selected from those already reported in the literature.

5.2.1.1 *M. tuberculosis* antigens analyzed

Both protective and TB pathologic antigens were included in this study. Secreted antigens Ag 85A, Ag 85B, Ag 85C, CFP-10 and ESAT-6 are known to enhance IFN-γ production [19,20]. Similarly, antigens Rv1818c, Rv3812 and Rv3018c from the PE/PPE [21] family are also known to increase IFN-γ secretion while *M. tb* 8.4, *M. tb*9.9A, 19 kDa protein and EsxG are known to harbor CTL epitopes [22]. Similarly, proteins of RD12 region (Rv2072c-Rv2075c) and RD13 region (Rv2645-Rv2660c) which are known to induce IL-10 and hence are instrumental in pathogenesis have also been included in this study [6].

The sequence of these antigens were retrieved from NCBI entrez Protein database at http://www.ncbi.nlm.nih.gov/protein

5.2.1.2 HLA alleles

Several class I alleles have been studied in order to understand their association with tuberculosis. These studies are based on the occurrence of the respective alleles found in tuberculosis patients and healthy controls. Those that are found more in tuberculosis patients are considered susceptible while those found more frequently in healthy controls are considered as protective. The alleles included in this study were based on various studies (Table 5.1).

Table 5.1: Protective and susceptible alleles based on molecular studies

Protective	Susceptible	Reference
HLA-A2	HLA-B62	Dubaniewicz et al., 2003
	HLA-B14	Ruggiero et al., 2004
	HLA-B35	Soto et al, 2007
HLA-B44	HLA-B60	Bothamley et al., 1999
HLA-A3, HLA-B44	HLA-A1	Balamurugan et al., 2004
HLA-A11	HLA-B40	Figueiredo et al., 2008
	HLA-B8	Selby et al, 1978
	HLA-B27	Zervas et al.,1987
	HLA-B7 HLA-B27	Nazirov et al., 1991
HLA-B52	HLA-B51	Vijaya Lakshmi et al., 2006
HLA-A11	HLA-B*4006(B61)	Raghavan et al., 2009

5.2.1.3 Prediction of epitopes with CTLpred

The CTLpred server allows the user to predict epitopes using quantitative matrix (QM), Support Vector Machine (SVM) and Artificial Neural Network (ANN) approaches. Sequences of the antigens obtained from NCBI were used as input sequence. The server allows the user to employ these approaches either individually or by combining ANN and SVM or performing a consensus prediction using ANN and SVM. The consensus approach and the combined approach increase the specificity and sensitivity of the prediction respectively. The user can vary the cutoff score for all prediction approaches. A consensus approach was used to predict the antigenic peptides with a cutoff score of 0.51 (default value) for ANN and 0.36 (default value) for SVM. The number of top scoring peptides to be displayed can also be chosen. We opted to analyze the top three scoring peptides and the results obtained (unpublished) include the nanomer peptides in a descending order of their score. The user is also given the choice of selecting a particular matrix i.e nHLAPred or Propred for finding the HLA restriction of the peptides. We chose Propred to identify the HLA alleles that could bind to the respective epitopes. CTLpred can be accessed freely from URL http://www.imtech.res.in/raghava/ctlpred (Bhasin et al., 2004).

5.2.2 Patient and Healthy controls

Tuberculosis patients with roentgenographic findings (chest X-ray) consistent with TB and positive sputum positive for AFB (acid fast bacilli) and negative human immunodeficiency virus serologic test were enrolled for this study. Asymptomatic healthy controls were also included in the study. Two patients and two controls were HLA-B44 positive whereas one Patient and one control were HLA-B44 negative. All procedures used in the study were approved by Institutional Ethics Committee for Human Research of the Faculty of Science, M. S. University of Baroda. Written informed consent was obtained from all subjects.

5.2.3 RNA isolation and cDNA synthesis

Peripheral blood mononuclear cells (PBMCs) from blood samples of patients (n=5) and controls (n=5) residing in Vadodara were isolated by Histopaque density gradient centrifugation. Total RNA was extracted using total RNA extraction kit (as described in chapter 2).The quality of the RNA samples was assessed by inspecting the 28S and 18S bands following agarose gel electrophoresis. A quantity of 5µg of each RNA sample was used with Oligo (dT18) for cDNA synthesis in a 20µl system using an M-MuLV RT-PCR kit (Bangalore Genei) following the manufacturer's instructions. Each of the cDNA preparations was then amplified for thirty cycles in a thermal cycler (Eppendorf, Hamburg, Germany) with β-actin-specific primers (forward, 5'-AGCGGGAAATCGTGCGTGACA- 3'; reverse, 5'-CGCAACTAAGTCATAGTCCG-3', generating an amplicon of 536 bp) by taking 1 µl of the cDNA in a 12·5µl system. This was used as a control for the synthesis of cDNA.

5.2.4 PCR amplification and identification of HLA-B allele

HLA-B specific primers and amplification conditions were used as described earlier (Akatsuka,2002). Control for checking genomic DNA contamination included amplification of the total RNA without reverse transcription which did not give any amplicon (results not shown). PCR products separated on a 2% agarose gel were stained with ethidium bromide (0·5mg/ml). The amplicon obtained was eluted and cloned in T

cloning vector using (Fermentas, USA). The clones were then sent for sequencing to Banglore genei. The sequence obtained was then used to identify the allele using nucleotide blast tool in IMGT/ HLA database (http://www.ebi.ac.uk/ipd/imgt/hla/) web site.

Two patients and two controls that were identified to be HLA-B*44 positive. These individuals were again confirmed as HLA-B*44 by using SSP primers (Unipath laboratory, Ahemdabad). Another patient and control was identified as HLA-B*44 negative individuals.

5.2.5 Prediction of epitopes with Propred I and CTLpred

The epitopes were predicted on the basis of highest scoring peptides by Propred I and confimed by CTLpred epitope prection tool used in the study. Sequences of the antigens obtained from NCBI were used as input sequence for both the prediction tools. For Propred I the peptides were predicted at threshold value of 4% and the proteosome and immunoproteosome filters were kept on. The CTLpred bioinformatic tool was used as mentioned above. The combined approach which includes epitopes predicted by ANN and SVM was employed to predict the epitopes. The cutoff score was 0.51 (default value) for ANN and 0.36 (default value) for SVM.

5.2.6 Cell lines

MHC class-I deficient 721.221 (B-LCL) transfected with HLA-B*4403 was kindly provided by Derin Keskin (Harvard University, USA). The cell line was maintained Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 10mM-non-essential amino acids, 1mM-sodium pyruvate and gentamicin (50mg/ml). The cell line was grown at 37°C in a humidified atmosphere containing 5% CO2–95% air atmosphere.

5.2.7 Expansion and purification of effector T cell population

PBMC from patients and controls were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). PBMCs were resuspended in RPMI with 10% FCS

along with 25ng/ml IL-7 (Peprotech) and stimulated with ConA (10µg/ml) and peptides (10µg/ml) respectively. On third day 20µg/ml IL-2 was added. The media was then changed every third day having the same concentration of IL-2. The cells were maintained for two weeks following which the T cells were purified using nylon wool. The columns were prepared using 2ml syringe containing 0.1 grams nylon wool, which was then autoclaved for maintaining sterile conditions. Before use, the columns were equilibrated by washing with 10ml RPMI tissue culture media. The nonadherent cells from the wells were carefully aspirated and 1.5 x 10^8 cells were resuspended in 1ml warm RPMI and subjected to nylon wool purification by loading onto the columns. The columns were then incubated for 30 mins at 37°C and 5% CO_2. The enriched T cells were then eluted by flushing the column with additional RPMI with 10% FCS.

5.2.8 Cytotoxicity assay

B-LCL was incubated in medium without G418 with 50µg/ml peptide. B-LCL incubated without peptide were used as negative control. Following overnight incubation the target cells were then incubated with 7.5µM calcein-AM (Sigma-Aldrich, USA) for 30 mins and then washed twice with PBS. Both effector and target cells were counted with tryphan blue dye prior to use for assessing their viability. The cytotoxicity assay was performed by incubating the effector and target cells (E:T) in the ratio 1:10, 1:20, 1:40 for 4 hours. 100 µl of the supernatants per well were harvested to measure fluorescence in a plate reader using 485nm excitation and 538nm emission by Spectramax M3 miroplate reader (molecular devices). The amount of calcein released is propotional to the CTL cytotoxicity (% specific lysis). The formula for % specific lysis is given by (test release – spontaneous release / maximum release – spontaneous release) * 100. Here spontaneous release is the amount of fluorescence released by target cells only and maximum release is the amount of fluorescence released by lysing the target cells with 2% Triton X-100. Data are reported as the mean of triplicate determinations.

5.2.9 Statistical analysis

The data obtained from in silico analysis was statistically analysed using unpaired Student's t test using GraphPad Prism 5 software. A p-value of less than 0.05 was considered to be statistically-significant and the results were expressed as mean ± SEM. The statistical analysis of CTL assay was done using one-way ANOVA with Dunnett's multiple comparison test. A p-value of less than 0.05 was considered to be statistically-significant.

5.3 RESULTS

5.3.1 *In silico* analysis of protective and pathologic tuberculosis antigens

For analysis, the protein sequence of selected antigens was submitted to CTLpred. CTLpred allows one to view the results onsite in a tabular form and also to choose the number of top scoring peptides to be displayed. The top three peptides were chosen for analysis. This result (onsite) shows the sequence of the peptides, the starting position and the score. CTLpred also states whether the peptide can be considered as epitope or non-epitope based on the comparison of the peptide scores for ANN and SVM with respect to default value of 0.51 and 0.36 for ANN and SVM respectively. The result of HLA restriction of these peptides using Propred matrix is displayed in Tables 5.2-5.4, where only the alleles that are known to be protective or susceptible are included. However there were some epitopes that did not bind to any of the selected HLA alleles and hence not shown in these tables. Also, in few cases such as ESAT-6 only two epitopes were predicted as HLA binders.

Table 5.2A and Table 5.3 show epitopes from protective antigens that may bind to various protective or susceptible alleles respectively. Similarly, table 5.2B and table 5.4 display epitopes from TB pathologic antigens that may bind to known protective or susceptible HLA alleles respectively.

When the protective antigens were queried against protective alleles, the total number of alleles predicted was not significantly different ($p=0.0923$) from that of susceptible alleles (Fig 5.1B). However, as seen from Figure 5.1A, the total number of epitopes from TB pathologic antigens predicted for the susceptible alleles significantly higher ($p = 0.006$) that for the protective alleles. Protective alleles HLA-A2 and HLA-A*1101 have five predicted epitopes of protective antigens each in contrast to seven and three predicted epitopes of TB pathologic antigen. Similarly, six epitopes each of protective antigens were predicted for HLA-A3 and HLA-B*4403 whereas seven epitopes, of TB pathologic antigens each. HLA-B*5201 was the only protective allele to have more

predicted epitopes of protective antigens (4 epitopes) than the TB pathologic antigen (2 epitopes) (Tables 5.2B & 5.4).

Analysis of susceptible alleles not only predicted a large number of epitopes of protective antigens (table 5.3) but also a high number of predicted epitopes of TB pathologic antigens (table 5.5). HLA-B*2705 had the highest number of total predicted epitopes (29 epitopes) with nineteen predicted epitopes of TB pathologic antigens and only ten of protective antigens. Similarly, HLA-B14 showed the second highest number of predicted epitopes (28 epitopes) including seven from protective antigens and the maximum of 21 epitopes from TB pathologic antigens. Nineteen epitopes of TB pathologic antigens were predicted for B7, whereas only eight were predicted for protective antigens. Similarly, thirteen and fifteen epitopes of TB pathologic antigens were predicted for HLA-B51 and HLA-B60 in contrast to seven and six epitopes respectively from protective antigens. Twelve epitopes of TB pathologic antigens were predicted for HLA-B8 and HLA-B61 each whereas only seven and five respectively for protective antigens. HLA-B62 had five predicted epitopes of protective and susceptible antigens each.

Table 5.2A CTLpred analysis of protective antigen association with Protective HLA-Alleles

HLA-A2	HLA-A3	HLA-A*1101	HLA-B*4403	HLA-B*5201
WPYWNEQLV-85C	AMGPTLIGL-85A	WLSANRAVK-85B	AAVPTTTVL-Rv1818c	FQGGGPHAV-85C
RQAGVQYSR-cfp10	WLSANRAVK-85B	RQAGVQYSR-cfp10	SELPAVAWV-Rv3018c	WPYWNEQLV-85C
AVVRFQEAA-cfp10	RQAGVQYSR-cfp10	SANPFPFLR-Rv3812	DPRSNLARF-Rv2350c	HTGPAPVIV-Rv3018c
SELPAVAWV-Rv3018c	SANPFPFLR-Rv3812	ATKDGSHYK-19kda	CENPGIREF-Rv2350c	RVPPRPYVM-*M. tb*39
AYVPYVAWL-Rv3018c	ATKDGSHYK-19kda	RVPPRPYVM-*M. tb*39	AAAAKVNTL-ExsG	
	NVNGVTLGY-19kda		AAASTYTGF-ExsG	

Table 5.2B CTLpred analysis of TB pathologic antigen association with Protective HLA-Alleles

HLA-A2	HLA-A3	HLA-A*1101	HLA-B*4403	HLA-B*5201
ALRPMFVAL-Rv2073c	ALRPMFVAL-Rv2073c	RVVDGRVLR-Rv2645	AELRRANAI-Rv2648	RSWPGCTAV-Rv2072c
TLDDGRRQL-Rv2645	RVVDGRVLR-Rv2645	WVDWFNHRR-Rv2649	REGDVIVRV-Rv2651c	STWAGFAYV-Rv2649
AKADRRIEL-Rv2646	TLRHRYATR-Rv2646		GERVRAQVL-Rv2652c	
STWAGFAYV-Rv2649	WVDWFNHRR-Rv2649		AESHGVAAV-RV2654c	
VLVDNAFRV-Rv2650c	VLVDNAFRV-Rv2650c		TEDRAPATV-Rv2656c	
SEAAEYLAV-Rv2657c	ALCLRLSQL-Rv2658c		SEAAEYLAV-Rv2657c	
ALCLRLSQL-Rv2658c	VVAPSQFTF-Rv2660c		CAILGLNQF-Rv2660c	

Table 5.3 CTLpred analysis of protective antigen association with susceptible HLA-Alleles

HLA-A1	HLA-B7	HLA-B8	HLA-B14	HLA-B*2705	HLA-B51	HLA-B60	HLA-B61	HLA-B62
AADEVSAAM-Rv1818c	AMGPTLIGL-85A	HVKPTGSAV-85A	QRNDPLLNV-85A	QRNDPLLNV-85A	WLSANRAVK-85B	RPGLPVEYL-85B	WPYWNEQLV-85C	FQGGGPHAV-85C
SANPFPFLR-Rv3812	RPGLPVEYL-85B	RPGLPVEYL-85B	AMGPTLIGL-85A	AMGPTLIGL-85A	IYAGSLSAL-85B	TATELNNAL-esat6	AADDVSIAV-Rv3812	NVASGTAGF-Rv1818c
AADDVSIAV-Rv3812	WPYWNEQLV-85C	TATELNNAL-esat6	KRNDPMVQI-85C	WLSANRAVK-85B	RPGLPVEYL-85B	AANKQKQEL-cfp10	SELPAVAWV-Rv3018c	NVNGVTLGY-19kda
HTGPAPVIV-Rv3018c	AANKQKQEL-cfp10	AANKQKQEL-cfp10	AAVPTTTVL-Rv1818c	RPGLPVEYL-85B	FQGGGPHAV-85C	AAVPTTTVL-Rv1818c	HTGPAPVIV-Rv3018c	HQAIVRDVL-9.9a
NVNGVTLGY-19kda	AAVPTTTVL-Rv1818c	AAVPTTTVL-Rv1818c	HQAIVRDVL-9.9a	KRNDPMVQI-85C	WPYWNEQLV-85C	SELPAVAWV-Rv3018c	AANQLMNNV-*M. tb*39	AAASTYTGF-ExsG
	APPPQRAAM -*M. tb*8.4	RVPPRPYVM-*M. tb*39	RVPPRPYVM-*M. tb*39	FQGGGPHAV-85C	APPPQRAAM -*M. tb*8.4	HQAIVRDVL-9.9a		
	HQAIVRDVL-9.9a	AAHARFVAA-ExsG	LRVPPRPYV-*M. tb*39	RQAGVQYSR-cfp10	LRVPPRPYV-*M. tb*39			
	RVPPRPYVM-*M. tb*39			KRGLTVAVA-19kda				
				HQAIVRDVL-9.9a				
				LRVPPRPYV-*M. tb*39				

Table 5.4 CTLpred analysis of TB pathologic antigen association with susceptible HLA-Alleles

HLA-A1	HLA-B7	HLA-B8	HLA-B14	HLA-B*2705	HLA-B51	HLA-B60	HLA-B61	HLA-B62
TLDDGRRQL-Rv2645	AARPSVIFL-Rv2072c	AARPSVIFL-Rv2072c	IRVLTLAAL-Rv2072c	RSWPGCTAV-Rv2072c	IRVLTLAAL-Rv2072c	AARPSVIFL-Rv2072c	RSWPGCTAV-Rv2072c	ALRPMFVAL-Rv2073c
AKADRRIEL-Rv2646	ALRPMFVAL-Rv2073c	ALRPMFVAL-Rv2073c	IRVRRANYV-Rv2073c	IRVLTLAAL-Rv2072c	IRVRRANYV-Rv2073c	AANKQKQEL-Rv2073c	TPRPNPRRV-Rv2074	VLGIGPAAA-Rv2645
STWAGFAYV-Rv2649	AANKQKQEL-Rv2073c	TPRPNPRRV-Rv2074	RPNPRRVVI-Rv2074	IRVRRANYV-Rv2073c	TPRPNPRRV-Rv2074	RASGARAVL-Rv2075c	NPRRVVIEV-Rv2074	VLVDNAFRV-Rv2650c
SEAAEYLAV-Rv2657c	TPRPNPRRV-Rv2074	RPNPRRVVI-Rv2074	RASGARAVL-Rv2075c	ALRPMFVAL-Rv2073c	NPRRVVIEV-Rv2074	AELRRANAI-Rv2648	AELRRANAI-Rv2648	ALCLRLSQL-Rv2658c
ALCLRLSQL-Rv2658c	RPNPRRVVI-Rv2074	RASGARAVL-Rv2075c	TLDDGRRQL-Rv2645	NPRRVVIEV-Rv2074	RPNPRRVVI-Rv2074	REGDVIVRV-Rv2651c	STWAGFAYV-Rv2649	CAILGLNQF-Rv2660c
	RASGARAVL-Rv2075c	TLRHRYATR-Rv2646	RRIELMIRL-Rv2646	RASGARAVL-Rv2075c	WVDWFNHRR-Rv2649	AESHGVAAV-RV2654c	REGDVIVRV-Rv2651c	
	GERVRAQVL-Rv2652c	AKADRRIEL-Rv2646	AKADRRIEL-Rv2646	RRIELMIRL-Rv2646	WRSIEDVEL-Rv2649	ESHGVAAVL-RV2654c	GERVRAQVL-Rv2652c	
	DPKPGKRRV-Rv2652c	RPKAKQRQR-Rv2647	RKDFTPSEL-Rv2647	WRSIEDVEL-Rv2649	VLVDNAFRV-Rv2650c	AAVELARAL-RV2654c	DPKPGKRRV-Rv2652c	
	RVVPELAAL-Rv2652c	DPKPGKRRV-Rv2652c	WRSIEDVEL-Rv2649	REGDVIVRV-Rv2651c	DPKPGKRRV-Rv2652c	AESHGVAAV-Rv2654c	AESHGVAAV-RV2654c	
	APRRNRVGR-Rv2653c	AAVELARAL-RV2654c	DRVGSTVEL-Rv2650c	GERVRAQVL-Rv2652c	RPAGGHIQM-Rv2658c	ESHGVAAVL-Rv2654c	AESHGVAAV-Rv2654c	
	ESHGVAAVL-RV2654c	ESHGVAAVL-Rv2654c	TRYPVGRAV-Rv2651c	RVVPELAAL-Rv2652c	MRYGELTEL-Rv2659c	AAVELARAL-Rv2654c	TEDRAPATV-Rv2656c	
	AAVELARAL-RV2654c	RPDLRVHDL-Rv2659c	SRSLAEARL-Rv2651c	RRAQRQRDL-Rv2653c	RPDLRVHDL-Rv2659c	TEDRAPATV-Rv2656c	SEAAEYLAV-Rv2657c	
	ESHGVAAVL-Rv2654c		RRAQRQRDL-Rv2653c	RRRDAYIRR-Rv2656c	VVAPSQFTF-Rv2660c	RSGTRLVRL-Rv2657c		
	AAVELARAL-Rv2654c		ESHGVAAVL-RV2654c	RRDAYIRRV-Rv2656c		SEAAEYLAV-Rv2657c		
	RSGTRLVRL-Rv2657c		AAVELARAL-RV2654c	RRYITISEA-Rv2657c		RPDLRVHDL-Rv2659c		
	ALCLRLSQL-Rv2658c		RRYITISEA-Rv2657c	ALCLRLSQL-Rv2658c				
	RPAGGHIQM-Rv2658c		RSGTRLVRL-Rv2657c	MRYGELTEL-Rv2659c				
	TLAELMQRL-Rv2659c		ALCLRLSQL-Rv2658c	TLAELMQRL-Rv2659c				
	RPDLRVHDL-Rv2659c		RHVIPFSAL-Rv2658c	RPDLRVHDL-Rv2659c				
			MRYGELTEL-Rv2659c					
			RPDLRVHDL-Rv2659c					

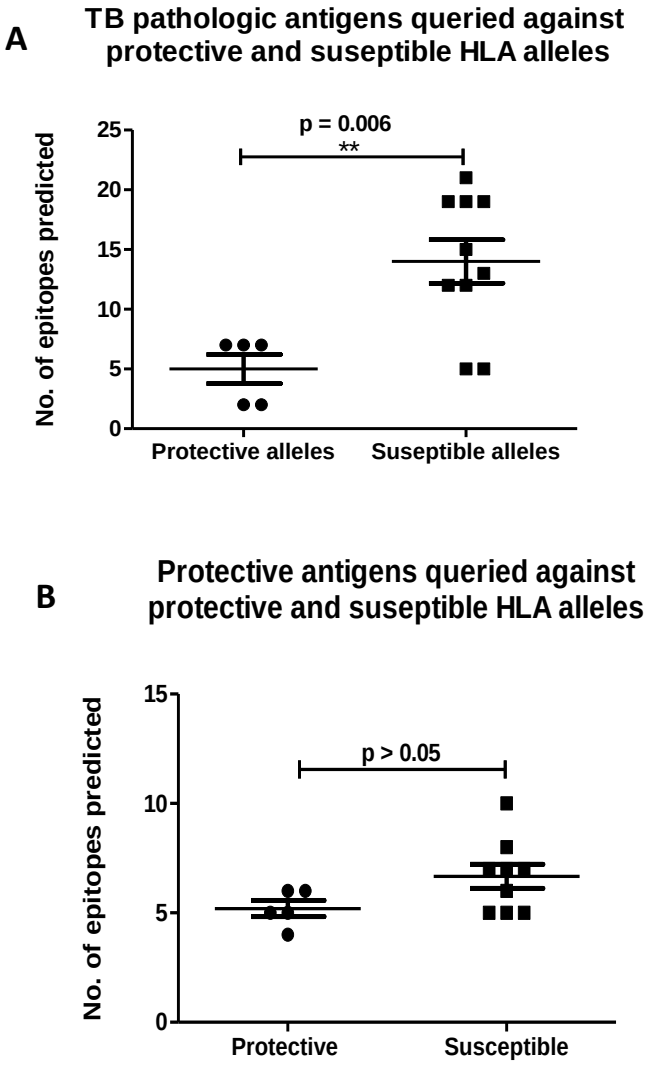


Figure 5.1 Graph showing number of predicted epitopes of TB pathologic antigens and protective antigens when queried against protective and susceptible HLA alleles using CTLpred. The sequence of antigens of *M.tuberculosis* which are known to contribute to disease pathology (TB pathologic antigen, Fig. 5.1 A) or induce protective immunity (Protective antigens, Fig. 5.1 B) were queried against HLA alleles known to be protective or susceptible in tuberculosis using CTLpred bioinformatic tool. The number of epitopes for protective and susceptible alleles respectively were plotted on graph. Results were expressed as mean ± SEM. The data was statistically analysed using unpaired Student's t test using GraphPad Prism 5 software. A p-value of less than 0.05 was considered to be statistically-significant.

5.3.2 HLA identification for various individuals included in the CTL study

Characterisation of the HLA genes was done by HLA specific PCR amplification of the cDNA prepared from various individuals. The amplicons thereby generated were then cloned and sequenced following which the HLA type was identified by using nucleotide blast tool in IMGT/HLA database. Furthermore, the genomic DNA from each individual was also sent for identification and the allele was reconfirmed by SSP analysis. As shown in Figure 5.2, the total RNA extracted from PBMCs of various individuals was of good quality and therefore could be used for subsequent cDNA preparations without any ambiguity.

The cDNA prepared from each of the RNA samples was then subjected to PCR using β-actin specific primers. The amplicon generated was then analysed by 0.8% agarose gel electrophoresis. Figure 5.2B shows the β-actin specific amplicons obtained from each of the cDNA samples, reflecting upon the excellent quality of the various cDNA preparations.

Following confirmation of the quality of the cDNA preparations, the same was used for amplifying the HLA-B genes by using specific primers. As shown in Figure 5.2C each sample showed the presence of an amplicon corresponding to the HLA-B gene. This was further confirmed by cloning and sequencing the respective amplicons. The identification of each HLA-B gene was done using nucleotide blast tool in IMGT/ HLA database and is provided in Table 5.5A. As can be seen from Table 5.5A, HLA-B 44 is the most frequent HLA-B type amongst both the patients and controls. Since the frequency of allele HLA-B*4403 found in both the groups and the frequency was high, the patients and controls of this allele was selected for further study. The allele identification of was again confirmed using SSP primers (Unipath Laboratory, Ahmedabad, India).

5.3.3 Peptide prediction for HLA-B*4403 from various M. tuberculosis antigens

The HLA-B*4403 restricted epitopes was identified for *M. tuberculosis* antigens Ag 85B, CFP-10, plc A, plc B, plc C and Rv2074 using bioinformatic tools Propred-I and CTLpred (Table 5.5B).

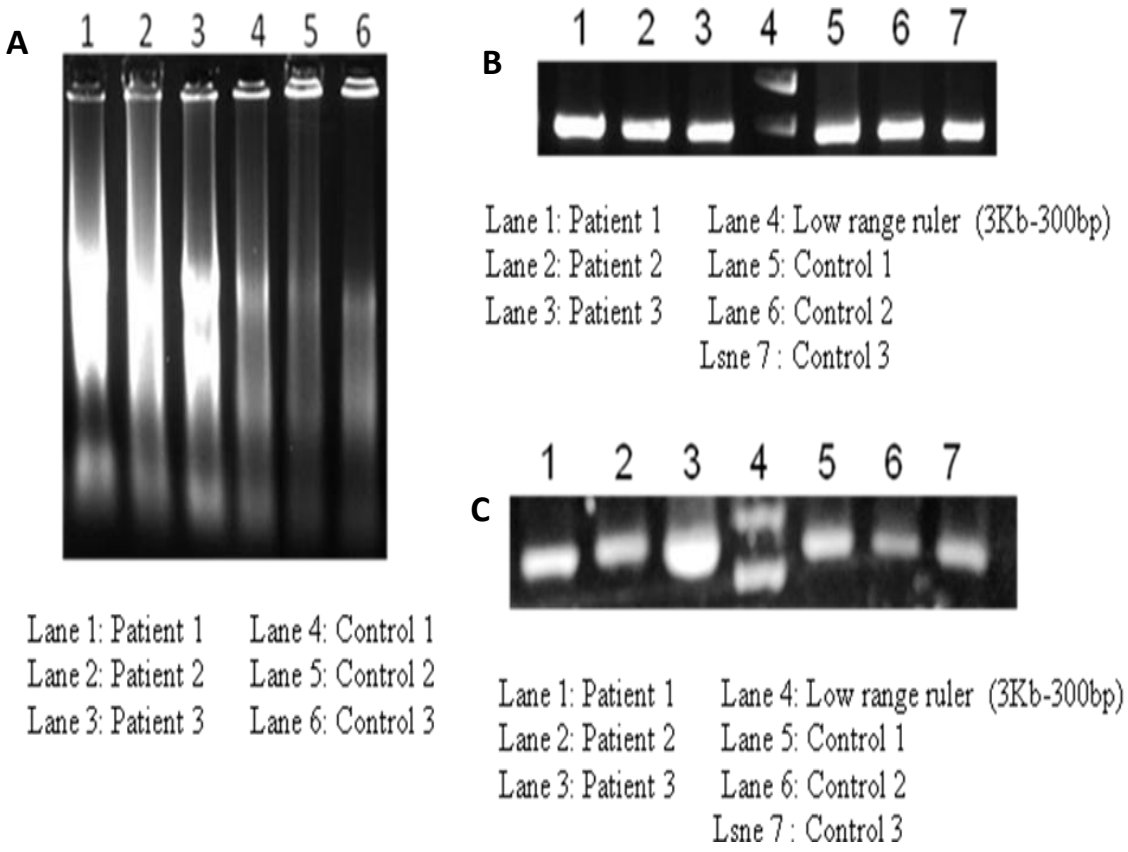


Figure 5.2 HLA gene amplification. Figure shows representative gel picture of 0.8% agarose gel with ethidium bromide stained samples of (A) RNA extracted from PBMC of patients and controls. (B) β actin was amplified for cDNA both the groups and then (C) HLA-B gene was identified using sequence specific primers.

Table 5.5A Characteristics of patients and controls included in the study. The average (Mean ± SD) age of the patients was 27.4 ± 5.1 and controls was 28.4 ± 2.8. The sputum positive results indicate : + - 1-9 bacilli/100 fields , ++ - 1-9 bacilli/10 fields, +++ - 1-9/field, ++++ - > 9/field.

Patients						Controls			
Sr. No.	Sex/ Age	Sputum AFB	HIV	HLA-B allele identified	% identity	Sr. No.	Sex/ Age	HLA-B allele Identified	% identity
1	M/19	++	Neg.	HLA-B*51022	97%	1.	M/29	HLA-B*44032	98%
2	M/30	++++	Neg.	HLA-B*44032	98%	2.	M/26	HLA-B*52	99%
3	M/26	++	Neg.	HLAB*520102	99%	3.	F/30	HLA-B*52012	97%
4	F/30	+++	Neg.	HLA-B*44032	99%	4.	F/25	HLA-B*44032	96%
5	F/32	+++	Neg.	HLA-B*44031	97%	5.	F/32	HLA-B*4421	99%

Table 5.5B HLA-B*4403 restricted peptides prediction. The peptides for HLA-B*4403 was predicted based on consensus between bioinformatic tools Propred-I and CTLpred. The peptides were selected on the basis of highest scoring peptides that were predicted as epitopes by both the bioinformatic tools

Sr. No.	Antigen	Predicted Peptide	Start Position	Propred-I Score	CTLpred Score ANN/SVM
1.	Ag 85A	AAAVVLPGL	20	18	0.84/0.46
2.	CFP-10	TAAQAAVVR	49	6	0.36/0.40
3.	plc A	QDIPIHYLL	157	36	0.61/0.36
4.	plc B	RDLPIHYLL	160	60.75	0.80/0.22
5.	plc C	AGAGAAVL	11	120	0.51/0.193
6.	Rv 2074	IEVQIERVL	120	720	0.87/0.87

5.3.4 Cytotoxicity assay

Cytotoxicity assay was carried out using effector cells generated from PBMCs of HLA-B*4403 positive, two patients and two control individuals in the presence of respective peptides. HLA-B*4403 transfected B-LCL cell line was stimulated with peptide and stained with calcein and was subsequently used as target cells. % specific lysis was calculated from the O.D. of calcein released into the medium. The peptides and target cells were incubated at the E:T ratios of 10:1, 20:1 and 40:1 respectively. Figure 5.3 presents the percentage of lysis of HLA-B specific B-LCL target cells by various effector cell populations in a peptide specific manner. As can be seen from the figure, all the peptides except the peptide derived from Rv2074 resulted in recognition of target cells leading to high CTL activity. Among the phospholipase family, significantly higher CTL activity can be seen in the case of plc C peptide than in the case of plc A or plc B peptide. Peptides of protective antigens 85A and CFP-10 gave significantly high CTL activity. However, peptide derived from Rv2074 did show any significant cytotoxic activity.

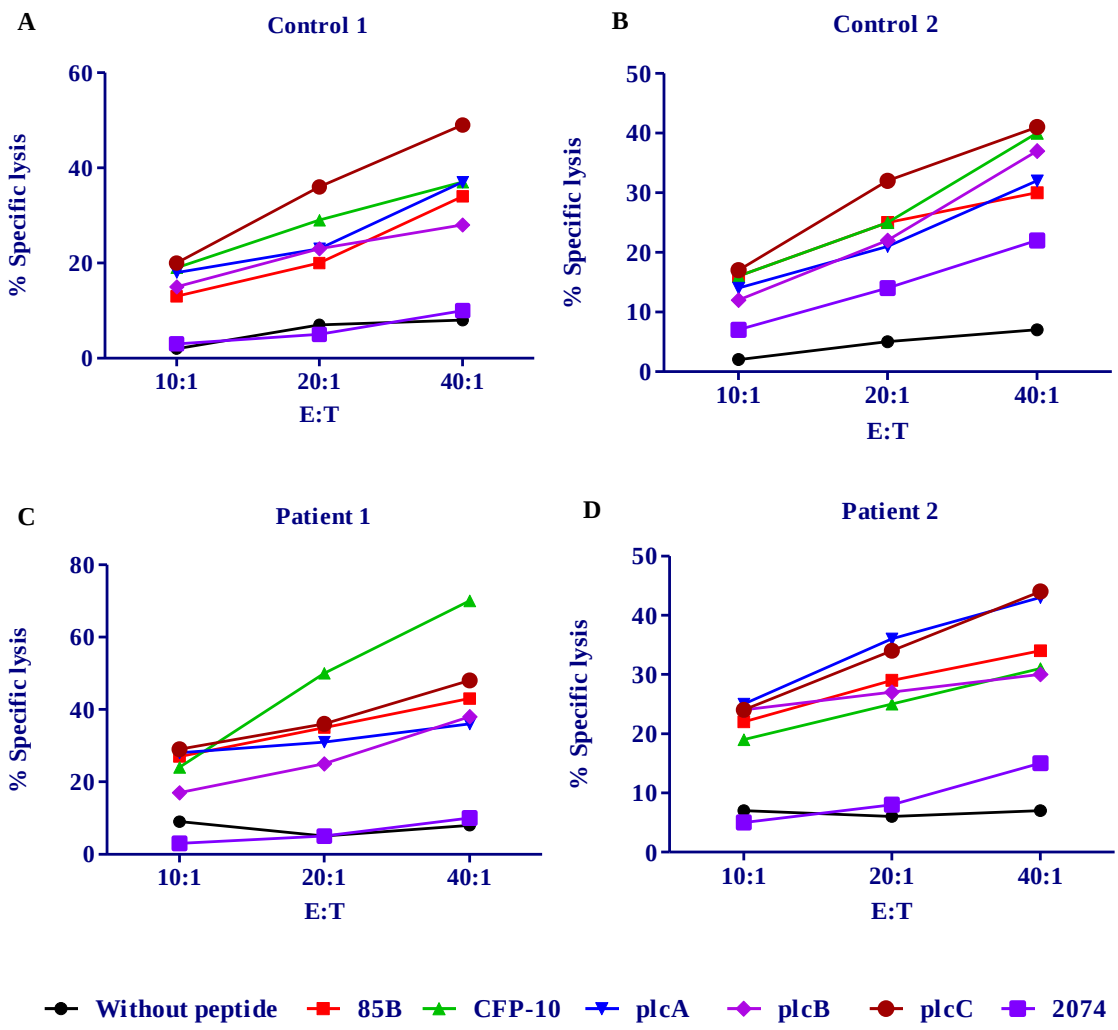


Figure 5.3 CTL assay. Graph showing % specific lysis of HLA-B*4403 transfected B-LCL target cells by effector T lymphocytes of HLA-B*4403 positive patients (A &C) and controls (B & D) and HLA-B*4403 negative patient (E) and control (F) respectively. Effector T lymphocytes were generated following stimulation of PBMCs with respective peptide and purified by nylon wool. The effector and calcein stained target cells were incubated without peptide or with respective peptides (Ag 85B, CFP-10, plc A, plc B, plc C and Rv 2074). The effector and target cells were then co-incubated in the ratio of 10:1, 20:1 and 40:1 ratio for 4 hrs the O.D. was measured and calcein released into the medium was estimated.

5.4 DISCUSSION

Several studies have highlighted the correlation of HLA genes with susceptibility to tuberculosis (Table 5.1). The present study was carried out to analyze *M. tb* specific epitopes in the context of protective and susceptible HLA class I alleles. CTLpred was the bioinformatic tool used for this purpose. CTLpred is based on quantitative matrix (QM), and machine learning techniques like Support Vector Machine (SVM) and Artificial Neural Network (ANN). QM quantifies each amino acid at each position and is simple to use. However since it does not take into account the neighboring residue effects within the peptide it only predicts good binders but does not propose binding motif i.e. it ignores the contribution of overall peptide structure to binding. In contrast ANN can not only generalize from input data and tolerate noise and errors in data but also deal with non-linear problems. Moreover, ANN's are based on structural risk minimization. SVM's can predict epitopes with more accuracy than ANN's, whereas the combined and consensus approaches can predict epitopes with more accuracy than the individual approaches. In case of the combined approach, the sensitivity increases while in consensus approach the specificity increases. We have used the consensus approach to increase the specificity of our analysis (Bhasin and Raghava, 2004).

In the present study both protective and susceptible antigens have been included. Ag 85A, Ag 85B, Ag 85C, CFP-10 and ESAT-6 are the secreted antigens that have been studied extensively and are known to produce IFN-γ (Lim et al., 1999, Hasan et al., 2009). Among these, antigens like Ag 85A, Ag85B, CFP-10 and ESAT-6 have also been used for vaccine trials (Martin, 2005). PE/PPE family of proteins includes Rv1818c, Rv3812 and Rv3018c which are also known to increase IFN-γ production and have been selected for analysis (Chaitra et al., 2008). Various RD regions which were studied by Al-Attiyah and Mustafa (2008) where it was observed that peptide pools of RD12 region and RD13 region could induce IL-10 production and hence play a role in pathogenesis of the disease have also been included in our study.

Contini et al., (2008) in their study on *in silico* selection of Class II specific *M. tb* epitopes from whole genome observed that lesser number of epitopes bound to

susceptible alleles than the protective alleles. In contrast to this in our study we observed that the total number of epitopes that bound to TB susceptible alleles like HLA-B14 (28 epitopes) and HLA-B*2705 (29 epitopes) were significantly higher ($p = 0.0329$) in comparison to the protective alleles like HLA-B*52 (6 epitopes) and –B*4403 (13 epitopes). (Table 5.2A, 5.2B, 5.3 and 5.4). This was in spite of the fact that epitopes were derived from antigens that are both protective (antigen 85A and -85B) as well as TB pathologic (Rv2072c and Rv2645). The contrasting findings may reflect the relative importance of CTLs versus T- helper cells. It is possible that CTLs are more important in TB and hence the increased number of CTL epitopes being associated with susceptible allele in our study also reiterates this fact. In addition, it was also observed that when TB pathologic antigens are queried against all HLA alleles, no or very few peptides of RD12 region (Rv2072c-Rv2075c) and some epitopes of RD13 region (Rv2645-Rv2660c) are predicted to bind to protective HLA's while several epitopes are predicted to bind to susceptible HLA. This was observed in case of HLA-A as well as HLA-B. For example the protective Ag85A, had only one epitope that was predicted for only one protective allele i.e. HLA-A3. Where as in case of susceptible allele all the three epitopes i.e. AMGPTLIGL (for HLA-B7, HLA-B14 and HLA-B*2705) QRNDPLLNV (for HLA-B14 and HLA-B*2705) and HVKPTGSAV (for HLA-B8) were predicted to bind to various susceptible alleles. When protective antigens are queried against all HLA alleles they are shown to bind to protective as well as susceptible HLA alleles (Table 5.2A and 5.3). Hence it appears that only peptides that show binding to protective HLA alleles are probably protective and others are not.

Bothamley (1999) observed that there is an increased presence of HLA-B60 in smear positive patients and HLA-B44 in healthy controls. In attempting to validate this, our study showed the same number (six) of epitopes derived from protective antigens predicted for HLA-B60 (susceptible) as well as HLA-B*4403 (protective). Whereas in the case of disease enhancing antigens of RD12 and RD13 region, fifteen epitopes were predicted to bind to HLA-B60 in contrast to seven epitopes for HLA-B*4403. The propensity of some alleles towards disease could be due to their ability to induce IL-10, as seen in other studies where regulatory CTLs have been implicated (Gilliet and Liu,

2002). Similar association was also seen in the case of HLA-B51 (susceptible allele) and HLA-B52 (protective allele). In this case, seven and four epitopes of protective antigen were predicted to bind to HLA-B51 and HLA-B52 respectively. Whereas in case of disease pathogenesis antigens, five epitopes of RD12 and eight epitopes of RD13 were predicted to bind to HLA-B51. In contrast to this only one epitope of RD12 and RD13 region each were predicted in case of HLA-B52. These observations are in agreement with those made in a study by Vijaya Lakshmi et al. where the increased incidence of HLA-B52 in healthy individuals and HLA-B51 in TB and HIV-TB patients was observed (Lakshmi et al., 2006).

In case of protective alleles, it was observed that only one epitope, ALRPMFVAL from Rv2073c was found in case of HLA-A2, HLA-A3 and RSWPGCTAV from Rv2072c for HLA-B*5201 and no epitopes were predicted to bind to HLA-A*1101 and HLA-B*4403 when RD12 region was analyzed. Contrasting this, there was an increased number of epitopes of RD12 region being predicted for susceptible alleles like HLA-*B2705 (6 epitopes), HLA-B7 (6 epitopes), HLA-B8 (5 epitopes), HLA-B14 (4 epitopes) and HLA-B61 (3 epitopes) from RD12 region. We also observed the same contrast when RD13 region was analyzed. While only one epitope was predicted for protective alleles like HLA-B*5201, two for HLA-A*1101, six for HLA-A2 as well as HLA-A3 and seven for HLA-B*4403, on the other hand more than one epitope was predicted for all the susceptible alleles with a maximum of seventeen epitopes (HLA-B14) predicted for the same. Increased number of epitopes of RD13 region were predicted for HLA-*B2705 (13 epitopes), HLA-B7 (13 epitopes), HLA-B8 (7 epitopes) and HLA-B61 (9 epitopes).

Though ESAT-6 is considered to be highly immunogenic and is used in vaccine trials, we found that only two epitopes of ESAT-6 were predicted as HLA-binders by CTLpred and none predicted to bind to protective alleles. However, TATELNNAL did bind to susceptible alleles like HLA-B60 and HLA-B8. Hasan et al. (2009) observed that ESAT-6 induced IL-10 production was increased in tuberculosis patients. Smith et al. (2000) in their study also observed that CTLs from healthy controls as well as tuberculosis patients

recognized Ag85A and Ag85B. In contrast to this, ESAT-6 was recognized by CTLs of tuberculosis patients only and not healthy controls.

To summarize, the total number of epitopes predicted for both protective and susceptible alleles revealed that the number of epitopes of protective antigens predicted in both the groups are not significantly different (Fig 5.1B). However, the numbers of predicted epitopes of the TB pathologic antigens were found to be significantly high in susceptible alleles as compared to the protective alleles (Fig. 5.1A). The association of higher number of TB pathologic antigens with susceptible alleles appears to highlight the role of these HLA alleles in susceptibility and pathogenesis of the disease.

Following prediction of epitopes by bioinformatic tool, the next step was to determine the T cell activation by in vitro assays. Only those peptides that are shown to stimulate T cells can be considered to have some immunomodulatory function. For this several techniques are available to study peptide specific T cell responses. These techniques are based on the ability of T cells to recognize the antigen for e.g, including MHC tetramer staining, release of cytokines for e.g. enzyme-linked immunospot (ELISPOT) assay, intracellular cytokine staining (ICS) , proliferation in response to peptide for e.g. T cell proliferation assay and lysis of infected cells for e.g. cytotoxicity assay (such as lactate dehydrogenase release assay, 51Cr release assay and calcein release assay). We had used ELISPOT assay in chapter 2 to differentiate the immune status of tuberculosis patients from that of contacts and controls. In the present study, we used cytotoxicity assay by calcein release method. The advantage of using calcein for this purpose is that unlike 51chromium, calcein-acetoxymethyl (calcein-AM) is a non radioactive dye, has good retention in targets and low pH sensitivity and there the stain is not tranfered among cells.

This assay estimates the ability of the peptide to stimulate T cells to identify and lyse the cells presenting the peptide (or infected cells in case of tuberculosis infection) in MHC class I restricted manner. Hence, we first analysed the HLA-B alleles in both patients and controls. As shown in Table 5.5, we observed that HLA-B*4403 was found in both the groups. We therefore, used this allele to identify the candidate peptides using this allele

by Propred-I and confirmed it by using using other prediction tool, CTLpred. The antigens slelected include Ag85A and CFP-10 which are already known to induce cytotoxic T cell response. Kamath et al. (2004) in their study demonstrated that CD8+ T cells from *M. tuberculosis* infected mice not only recognized CFP-10 epitopes but also observed that among these cell s the IFN-γ producing CD8+ T cells preferentially migrated to lungs . Ag85A have been known to be a potent antigen for stimulating CD4+ as well as CD8+ T cell response (D'Souza et al., 2000; Tang et al., 2007). In addition to these we also included antigens from the phospholipase family i.e. phospholipase A, B and C. Phospholipase is known to be a virulence factor in organisms like *S. aureus, L. monocytogenes, P. aeruginosa* and *C. perfringens*. More recently, Raynaud et al. (2002) highlighted the role of phospholipase C in virulence of the tubercle bacillus. However, no study appears to have been done to identify its epitopes to generate cytotoxic T cell response. We also included Rv2074 antigen in our study, which is a member of RD12 region. The peptide pools of RD12 region are known to induce IL-10 (Th2 type) of response (Al-Attiyah and Mustafa, 2008). The epitopes of the above mentioned antigens were therefore predicted and used for CTL assay.

For performing CTL assay, two patients and two controls were identified positive for HLA-B*4403 by gene sequencing and using SSP method. The PBMCs' of these individuals were then used for the generating peptide specific effector cells by incubating them with respective peptides. Peptide laden HLA-B*4403 transfected B-LCL were stained with calcein and used as target cells. The CTL assay was carried out using effector:target ratio 1:10, 1:20 and 1:40. The calcein release was estimated and % specific lysis was calculated. It was observed that 1:40 ratio gave the highest % specific lysis. Further when we compared the lysis at this ratio of different peptides we found that all the peptides except Rv2074 displayed CTL response. In accordance to what is already known in the literature, the peptides of both Ag85B and CFP-10 showed more than 30% specific lysis. We also observed the % specific lysis of plcC peptide was consistently high in all the cases and showed significantly high % specific lysis in comparsion to other members of phopholipase C family (i.e. plc A and plcB) as well as Ag85B. CFP-10 and plcC showed similar % lysis. It is interesting to note that, while both plcC and plcB

genes are expressed at higher levels compared to plcA during the growth of virulent strain, peptide derived from all of them have more or less equal potential in activating the immune system as evidenced by the corresponding target cell lysis ability.

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LIST OF PUBLICATIONS

1. IL-10 modulates in vitro multinucleate giant cell formation in human tuberculosis.**PLoS One. 2013 Oct 17;8(10):e77680. doi: 10.1371/journal.pone.0077680.**

2. Differential expression of alpha-II spectrin in monocytes of tuberculosis patients **Int Immunopharmacol. 2013 Nov;17(3):759-62. doi: 10.1016/j.intimp.2013.09.005. Epub 2013 Sep 18.**

3. **Shrivastava P, Bagchi T.** In silico analysis of mycobacterim tuberculosis proteins to understand their role in susceptibility and protection. **Int J Biol Med Res. 2013; 4(4): 3607-3614**

- **Oral presentation on "Study on in vitro granuloma formation from monocytes of tubecrculosis patients and healthy controls" at the Indian Science Congress (Baroda chapter) – September 2012**

- **Poster presented, Shrivastava, P.J. and Bagchi, T. 'A study of FoxP3 versus pro- and anti-inflammatory cytokines in tuberculosis' presented at Society of Biological Chemists (SBC) at Lucknow, November 12-15, 2011.**