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

Tuberculosis is an ancient disease that still remains a global health concern with morbidity of 8.8 million people and mortality 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 ventilation and poor hygiene habits enhances the risk of transmission of tuberculosis by many folds. The increasing incidence of HIV infection further aggravates 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 microaggregate 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 immunosurveillance 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 TGF-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 stimulate 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 stimulated 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 the 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 tend 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, household 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 comparison to other members of phospholipase 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 predominant 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.