

## RELATIONSHIP BETWEEN HSP-65 AND IL-2, IL-10 PROFILE IN TB PATIENTS. IMPLICATION FOR VACCINE DESIGN: A PRELIMINARY STUDY

Anju S. Rajan<sup>1</sup>, Rajpal S. Kashyap<sup>1</sup>, Seema D. Shekhawat<sup>1</sup>, Aliabbas A Husain<sup>1</sup>, Hemant J Purohit<sup>2</sup>, Girdhar M Taori<sup>1</sup> and Hatim F Daginawala<sup>1\*</sup>

<sup>1</sup> Biochemistry Research Lab, Central India Institute of Medical Sciences, 88/2 Bajaj Nagar, Nagpur –440 010, INDIA

<sup>2</sup> Environmental Genomic Unit, National Environmental Engineering Research Institute (NEERI), CSIR, Nehru Marg, Nagpur–440 020, INDIA

### ABSTRACT

Tuberculosis (TB) is a serious infectious disease. *Bacillus Calmette Guerin* (BCG) only vaccine currently available has shown to offer limited protection against incidence of TB. Hence novel vaccines instead of BCG are required for protection against TB worldwide. Mounting evidence suggest that ideal vaccine against TB should drive efficient Th1 immune response. Hsp65 is a major immunoreactive protein and is supposed to be an important target for subunit vaccine development. In present study serum samples of TB patients were analyzed for Hsp 65 expression and also IL-2 and IL-10 cytokine profile was studied. Our preliminary results suggest that with an increase in Hsp 65 expression, level of IL-2 increased while that of IL-10 was found to get decreased. This relationship between Hsp65 and IL-10 and IL-2 cytokine profile suggest us that Hsp65 may modulate Th1 immune response and thus have a potential for vaccine design in near future. However, there are lots of future studies needed in in-vitro and animal models to prove the present concept.

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### KEY WORDS

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Corresponding author: Email: [hfd\\_ciims@rediffmail.com](mailto:hfd_ciims@rediffmail.com); Tel: +91-712-2233381/2236441; Fax: +91-0712-2236416

### [1] INTRODUCTION

Tuberculosis (TB) is a largest single infectious cause of human mortality [1-2]. The incidence of TB has remained high in most of the developing world and the disease is a public health problem. *Bacillus Calmette Guerin* (BCG) is among the worlds most widely used and only vaccine currently available against TB for use in humans. However due to lack of consistent protective efficacy in different parts of the world, it remains the most controversial vaccine in current use [3]. The development of a new improved TB vaccine is thus, a highly prioritized international research area. Vaccine potential of various novel antigens of *Mycobacterium tuberculosis* (MTB) including antigen 85 complexes and ESAT-6 have been tried by various investigators with encouraging results [3] However, no novel vaccine for clinical use has yet been developed in the world.

Other proteins, which have been proposed to have important immune target for subunit vaccine development are, heat shock protein (Hsp) family [4]. Hsp(s) are well-conserved and immunodominant antigens which elicit a cellular and humoral immune response and may play an important role in host defense against invading microorganisms and autoimmune disorders [5-6]. Among Hsp, 65 kD Hsp protein, is present in a

wide range of mycobacterial species and has been most intensively studied [7-9]. It is one of the major immunoreactive proteins of the mycobacteria [10-11]. It has been shown that Hsp(s) are potent activators of the innate immune system, capable of inducing proinflammatory cytokine production by the monocyte-macrophage system, and the activation and maturation of dendritic cells (antigen-presenting cells). which play an important role in host defense against TB.

Hsp 65 protein has been mapped at epitope level for both T and B cells recognition. MHC Class II specific CD4+, Th 1 cells recognize a single epitope in Hsp 65 indicating it to be an important candidate with immunotherapeutic and preventative vaccine potential [10-11]. The vaccination potential of this protein has also been explored in *M. habana* and MTB and was seen to confer protection against experimental challenges in mice [12].

Cytokines are important hormonal mediators, produced in tissues undergoing defense, growth and repair processes [12]. It is believed that immunity to TB is mediated by Th1 lymphocytes which activate antimicrobial macrophage

functions via the release of proinflammatory cytokines like IL-2, IL-12 and IFN- $\gamma$  [13]. Human monocytes, generally, respond to microbial infection by increasing the expression and secretion of such cytokines, which are involved in inflammatory responses. They play an important role in the modulation of the immune response to infection with MTB. tuberculosis. Thus current attempts to find a vaccine for TB are based on the assumption that it must drive Th1 immune response.

In the present study to justify our above hypothesis we examined expression of Hsp 65 in serum samples of TB patients and its co relationship with proinflammatory (IL-2) and anti-inflammatory (IL-10) cytokines.

## [II] MATERIALS AND METHODS

### 2.1. Study Subject

Serum samples of forty-two cases were analyzed in the present study for cytokine expression with respect to 65kD Hsp. All the TB patients (24 female; 18 males, age 15–65 years) had history and clinical findings compatible with diagnosis of TB. TB was confirmed if AFB staining and / or culture of sputum specimens were positive for MTB. Out of these, fourteen were both sputum smear and culture positive cases (Grade2+). Remaining twenty-eight patients were clinically suspected TB cases, where both AFB and culture tests were negative and diagnosis was made on the basis of clinical findings like, low grade fever, loss of appetite, abnormal chest X-ray, weight loss, night sweats, chronic cough with or without hemoptysis, chronic abdominal pain, past history of TB. Serum samples were obtained from all the patients before initiation of anti-koch treatment (AKT) and were stored at -20°C until they were tested. All patients included in the study were vaccinated with BCG. Samples were collected from all study groups for which patient's consent was obtained. The Central India Institute of Medical Sciences Ethical Committee, Nagpur, India approved the study. All the patients were grouped as follows:

### 2.2. Diagnostic Criteria

#### 2.2.1. Confirmed TB

TB was confirmed if AFB staining and /or culture of sputum specimens were positive for MTB.

#### 2.2.2. Clinically Suspected TB

When AFB staining and culture tests were negative, the diagnosis of TB was made on the following clinical observations:

a) Low grade fever, b) Loss of appetite, c) Abnormal Chest X-ray, d) Weight Loss, e) Night sweats, f) Chronic cough with or without hemoptysis, g) Chronic abdominal pain, h) Past history of TB

### 2.3. Specimens

Sputum specimens obtained from patients were digested and decontaminated with N-acetyl cystein (NALC) and 2% sodium hydroxide (NaLc-NaOH) solution and then processed for further investigations. Ziehl-Nielsen acid fast staining was used to confirm the presence of acid-fast bacilli. Venous blood was collected from all the patients as well as control subjects. Blood was allowed to clot and after centrifugation (1000xg, 10 min) serum was separated and stored at -20°C until use.

### 2.4. Antibodies

The 65kD monoclonal antibody was obtained from Colorado State University, USA through the TB Research Materials and Vaccine Testing Contract (NO1-AI-40091) derived from MTB, strain H37Rv, designated IT 13. The secondary antibody was rabbit anti rat IgG peroxidase conjugate obtained from Genei, Bangalore, India. IL-2 and IL-10 module Set were obtained from Bender Med Systems.

### 2.5. Estimation of 65kD Hsp antigen

Prior to patients sampling, the assay was standardized using different concentration of 65kD antigen (1-1000ng/ml) in Phosphate buffered saline pH 7.2 with 0.05% Tween 20 (PBS-T). After standardization, wells of flat-bottom microtiter plates were coated with 100  $\mu$ l of serum samples (1:200 dilutions in PBS-T) of selected groups and incubated for 90 min at 37°C. The wells were then washed with PBS-T and blocked with 100  $\mu$ l of 0.5% BSA in PBS-T at 37°C for 60 min. After blocking, monoclonal antibody against 65 kD Hsp antigen was added to all the wells (1:5,000 dilution in PBS-T) and incubated at 37°C for 60 min. The wells were washed with the PBS-T followed by addition of, 100  $\mu$ l of affinity purified anti-rat IgG-HRP conjugate (Genei, Bangalore, India) with 1:10,000 dilution in PBS-T, and incubated at 37°C for 60 min. After incubation the wells were washed extensively with PBS-T followed by addition of 100 $\mu$ l of TMB/H<sub>2</sub>O<sub>2</sub> substrate and incubated at room temperature for 10 min. The reaction was stopped with addition of 100  $\mu$ l of 2.5 N H<sub>2</sub>SO<sub>4</sub>. The absorbance of each well was read at 450 nm. Each sample was tested in triplicates.

### 2.6. Estimation of IL-2 and IL- 10

The serum samples were subjected to cytokine assay, IL-2 and IL-10 concentrations were measured by quantitative ELISA (IL-2 and IL-10 Module Set, Bender Med Systems). Flat-bottom micro titer plates were coated with 100  $\mu$ l of coating antibody solution and incubated at 2-8°C overnight. The wells were then washed once with 300  $\mu$ l PBS-T. 250  $\mu$ l of blocking buffer was added to each well and incubated at 37°C for 2 hrs. This was followed by washing and sample addition. 100 $\mu$ l sample and 50  $\mu$ l of diluted biotin conjugate (1  $\mu$ l/ml) were added to all the wells. The plates were incubated at room temperature (18°C - 25°C) on shaker for 2hrs. The wells were washed thrice with wash buffer followed by addition of 100  $\mu$ l diluted Streptavidin-HRP (0.2 $\mu$ l/ml) and incubation at room temperature for 1hr. After incubation the wells were washed thrice, 100 $\mu$ l TMB substrate was added to each well. After 10 min stop solution (100 $\mu$ l of 4N sulphuric acid) was added and absorbance was taken at 450nm.

### 2.7. Statistical Analysis

The Pearson's correlation amongst these along with their statistical significance is presented in Table-1 and Table-2. The correlation between IL-2 and 65kD in TB patients was found to be significant ( $p < 0.05$ ). Also the correlation between IL-10 and 65kD was found to be significant ( $p < 0.05$ ). Graphs were designed using Regression Scatter chart for IL-2, IL-10 and 65kD Hsp.

## [III] RESULTS

Serum samples of 42 TB cases were collected. All the samples were subjected to ELISA for detection of concentration of 65kD Hsp antigen in serum. The samples from the same group were then tested for IL-2 and IL-10 levels following the method given by Bender Med Systems. Relationship between 65kD Hsp, IL-2 and IL-10 was checked by plotting Regression

Table: 1. Demonstrates Pearson’s Correlation Coefficient for IL-2 and IL-10 with 65kD Hsp in serum samples of TB patients

Variable X	Variable Y	Correlation Coefficient r	Significance Level	95% Confidence interval for r	Positive/Negative Correlation
65kD	IL2	0.9602	<0.0001	0.9268 to 0.9786	Positive
65kD	IL10	-0.4358	0.0426	-0.7243 to -0.0174	Negative

graphs and Pearson’s Correlation Coefficient was also found in each case. P value less than 0.05 was considered to be significant.

Figure–1 shows the relationship between IL-2 and 65kD Hsp in serum samples of TB patients. The regression graph shows a significant increase in IL-2 with the increase in 65kD Hsp. A significant positive correlation between IL-2 and 65kD Hsp was found by Pearson’s Correlation Coefficient with  $P < 0.05$  [Table–1]. On the other hand significant decrease in IL-10 level with the increase in 65kD Hsp expression was noted in serum samples of TB patients as shown in Figure–2. A significant negative correlation was noted in this case and P value was found to be  $< 0.05$  [Table-1].

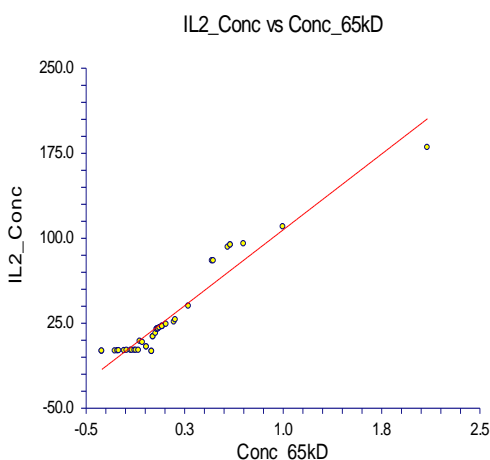


Fig: 1. Regressions between IL-2 and 65kD Hsp concentration in TB patients.

#### [IV] DISCUSSION

*Mycobacterium bovis* Bacillus Calmette Guerin (BCG) is the only vaccine available for clinical use but it has shown variable levels of efficacy against pulmonary TB, as demonstrated by several clinical trials. Given the increased public health importance of TB, the development of improved TB vaccines is an area that has been given priority by the WHO in its call for TB as a ‘Global Health Emergency’ [14].

Many antigens have been tried by various investigators for development of new TB vaccine candidate however still all are under experimental investigations. In the present study we have tried to establish a relationship between Hsp 65 and the increased level of IL-2 with decreasing level of IL-10, and postulated that it may have a role for vaccine design in future.

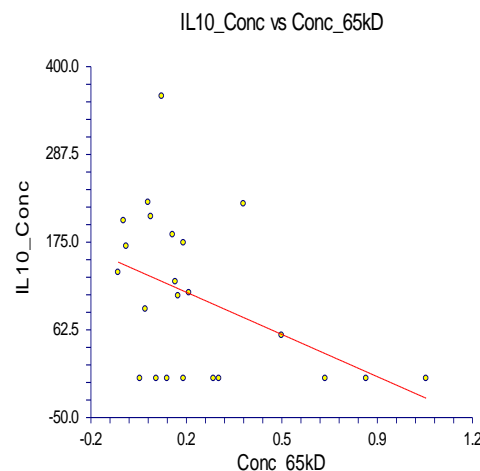


Fig: 2. Regressions between IL-10 and 65kD Hsp concentration in TB patients

As stated earlier, protective immunity against MTB depends on production of Th1 arm of immune response producing proinflammatory cytokines [15-16]. Our results demonstrate an increase in IL-2 levels with an increase in 65kD Hsp in tuberculosis. Thus indicating that 65kD Hsp enhance the expression of IL-2. IL-2 stimulates expansion and enhanced functional capacity of natural killer cells, which can eliminate intracellular MTB [17-18]. IL-2 strongly induces IFN-gamma production by murine splenocytes exposed to *M.bovis* BCG [19], and is a potent growth factor for CD4+ and CD8+ T cells, both of which contribute to immunity against TB [17].

In case of anti-inflammatory IL-10, our results demonstrated a decrease in IL-10 concentration with the increasing 65kD Hsp in patients infected with M.TB. Earlier Murray et al, demonstrated that IL-10 is an inhibitor of early mycobacterial clearance suggesting that IL-10 negatively regulates numerous macrophage functions as well as playing a role in down-

regulating the general inflammatory response, especially in conditions where an infection must be controlled through macrophage activity [19-20]. Similarly in another study done by Gloria et al, elevated pulmonary steady-state protein levels of IL-10, IFN-gamma, and bioactive TGF- $\beta$  were found in TB patients versus those in other lung disease patients and healthy volunteers. This observation suggests that the combined production of the immunosuppressant IL-10 and TGF- $\beta$ , as well as co expression of TGF- $\beta$  RI and RII (required for cellular response to TGF- $\beta$ ), may act to down-modulate host anti-MTB immunity and thereby allow uncontrolled bacterial replication and overt disease [21].

Progress in TB research has advanced as a result of the great amount of information obtained from the completion of the M.tb genome sequence. This has led to the rapid characterization and expression of MTB antigens with vaccine potential and Hsp 65 is one among them. Hsp 65 can elicit a strong delayed type hypersensitivity reaction in experimental animals infected with MTB [22]. Hsp 65 produced by *M.leprae* has been proved to be an efficient vaccine antigen against several pathologies including TB, leishmaniasis [23], diabetes, arthritis and cancer [24, 25, 26, 27]. The success of Hsp 65 vaccination in these different diseases reflects its effectiveness as an immunodominant antigen, which can induce Th1 response and CTL response against TB epitopes [26, 31, 32, 33]. Although Hsp are intracellular protein as they lack leader signal sequences that direct their secretion, still, they have been identified extracellularly in body fluids and

#### [IV] CONCLUSION

In conclusion, relationship between Hsp 65 and IL-10 and IL-2 cytokine profile suggest us that Hsp 65 may modulate Th1 immune response and thus have potential for vaccine design in near future.

#### FINANCIAL DISCLOSURE

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