6. BASIC RESEARCH

Completed Studies:

Development of luciferase reporter phages aiding diagnosis of latent tuberculosis

Background:

Early diagnosis of TB is essential for reducing the morbidity and mortality both in endemic and in HIV infected population. LRPs show promise in diagnostic microbiology. LRP constructs from D29 and TM4 used in conventional LRP assay are highly specific but lack in sensitivity as the lytic phage infection proceeds to host cell lysis. It was hypothesized that a luciferase reporter construct from temperate phage infecting *M. tuberculosis* would bring about a sustained light output leading to better sensitivity of LRP assay. Most of the TB cases among the HIV-infected population result from the reactivation of latent bacilli. LRP constructed with a dormancy inducible promoter driving the luciferase gene must be able to detect viable but not cultivable population of *M. tuberculosis* in clinical specimens.

Aim:

- To evaluate the performance of LRPs developed from temperate mycobacteriophage Che12 and TM4 derived temperature sensitive (TS) mutant, phAE159, with the conditional promoters, namely isocitrate lyase (icl) and alpha crystallin protein (acr)

Methods:

Light production kinetics of the LRP constructs was studied in *M. tuberculosis* $H_{37}Rv$. Luciferase activity of the LRP constructs was evaluated and compared with D29 based LRP phAE129 in both active and dormant (Wayne's dormant model) cultures of *M. tuberculosis* $H_{37}Rv$ and five clinical isolates of *M. tuberculosis.*

Results:

TM4 based phAETRC201 expressing firefly luciferase gene driven by hsp60 promoter and phAETRC202 with 'acr' promoter showed peak activity at 48 hours

(Figure 6.1). In the case of the Che12 construct phAETRC21 with 'icl' promoter, RLU reached maximum at 72 hours (Figure 6.2). All the three constructs exhibited detectable luciferase activity in actively growing cultures of *M. tuberculosis*. With the Wayne's dormant model phAETRC201 gave detectable light output with an increase in RLU by more than one log in all the strains tested, whereas phAETRC202 gave detectable RLU with only 4 of the isolates. PhAE129 gave detectable RLU in 3 strains tested (Figure 6.3). One of the clinical isolates resulted in detectable RLU only with phAETRC201 (TM4 based) and this was subsequently identified as a NTM.

Fig. 6.1: Light kinetics of TS mutant LRP constructs in *M.tuberculosis H*$_{37}$*Rv*

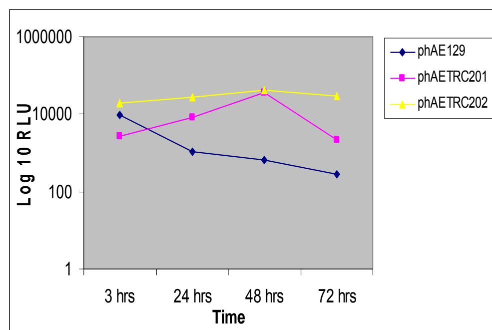


Fig. 6.2: Light kinetics of Che12 constructs in *M.tuberculosis* $H_{37}RV$

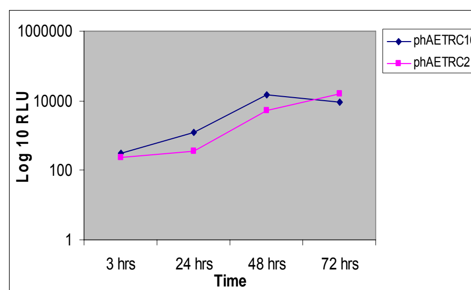
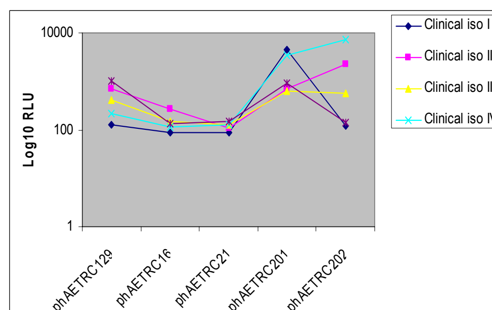


Fig. 6.3: Comparison of different LRPs in dormant cultures after 24 hours infection



Conclusion:

As the constructs exhibited detectable RLU at varying levels both in active and dormant models, it is recommended that use of all these constructs together should yield best results in TB diagnosis. Refining the assay format further should lead to an ideal assay to diagnose TB, to differentiate the species and to do direct DST.

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The status of the complement system in tuberculosis

Background:

The complement system serves as a major link between the innate and the adaptive immune system as well as facilitating production of appropriate immune response to an antigenic stimulus. It is known that mycobacteria and some of their antigens activate the complement system and the complement components are found in circulating immune complexes (CIC) in active TB. Further, levels of complement proteins and hemolytic complement have been shown to be higher in patients compared to healthy controls. Mice deficient in complement components are more susceptible to *M. tuberculosis* infection than control mice. These findings suggest that the complement system is involved in the pathogenesis of TB.

Aim:

- To investigate the status of the complement system in TB by documenting the quantitative changes that occur in this disease, functional aspects of the complement system and the effect of complement components on the host immune responses in pulmonary TB

Methods:

The levels of complement components and activation fragments in serum and complement receptors on peripheral blood mononuclear cells (PBMCs) were measured. The alteration of the functional capacity of the activated complement system in TB and the effect of complement on the immune responses against *M. tuberculosis* was studied.

Participants:

The study subjects comprised of 25 patients each with active, smear positive pulmonary TB and 25 patients who have completed the entire ATT regimen and 25 normal healthy volunteers.

Complement profile in TB:

The levels of complement components and their activation fragments were measured in serum using sandwich ELISA and the surface expression of complement receptors on PBMCs using flow cytometry.

Functional assay:

Functional characterization of complement activation was assessed by ELISA using coated IgM for the classical pathway and cell surface glycoproteins of mycobacteria for the alternative pathway.

Effect of the complement system on host immune response

The functional significance of the activated complement system on important host immune responses against *M. tuberculosis* was studied by analyzing the levels and components of CICs using ELISA, and the effect of *in vitro* addition of complement components on macrophage phagocytosis and apoptosis of mononuclear cells using flow cytometry.

Results:

Complement activation fragments like C3a, C4a, C5a and C3d were found to be higher in active TB patients followed by post treatment and control groups (Table 6.1).

Table 6.1: Serum complement profile showing the levels of C3, C3d, C3a, C4, C4a, factor B and C5a measured using ELISA (mean ± 95% CI).

	NHC (n = 25)	Treated TB (n = 25)	Active TB (n = 25)
C3 (AU/ml)	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.04
C3d (AU/ml) *	0.6 ± 0.1	1.0 ± 0.1 [a]	1.3 ± 0.1 [b, c]
C3a (ng/ml) *	11712.3 ± 2319.5	12949.7 ± 1948.8	21845.4 ± 1572.1 [b, c]
C4 (AU/ml)	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.04
C4a (ng/ml) *	1008.6 ± 452.6	1412.4 ± 576.7	2236.7 ± 244.5 [c +, b $]
Factor B (AU/ml)	0.8 ± 0.2	0.6 ± 0.2	0.8 ± 0.1
C5a (ng/ml) *	153.8 ± 50.3	691.7 ±192.4 [a]	1175.3 ± 253.8 [b, c £]

NHC-normal healthy controls; TR-treated PTB patients; UTR-untreated active PTB patients; n-number of individuals; CI-confidence interval

* Statistically significant differences (P<0.005) between groups (ANOVA).
[a] Differences are significant between NHC and TR groups (Post Hoc Bonferroni test).
[b] Differences are significant between NHC and UTR groups (Post Hoc Bonferroni test).
[c] Differences are significant between TR and UTR groups (Post Hoc Bonferroni test).
[#] P<0.001, [$] P=0.001, [+] P=0.038, [£] P=0.002 by Post Hoc Bonferroni test.

The levels of native complement proteins were comparable amongst the three study groups. However, high levels of complement split fragments were seen in patients compared to controls.

Under *in vitro* condition, the presence of complement is shown to modulate cytokine secretion by monocytes infected with *M. tuberculosis*.

Analysis of the components of CIC in pulmonary TB showed that the presence of complement components like C3d, C4c and C3 and the extent of immune complex precipitation was found to be higher in active TB patients compared to the other two groups examined.

Decreased expression of all four complement receptors on various cell types with different surface markers such as CD4, CD8, CD20 and CD14 was observed.

CR1 on erythrocytes were found to be decreased on erythrocytes of patients with active TB compared to treated patients and controls.

Conclusion:

The presence of complement is shown to augment the percentage of mononuclear cells undergoing apoptosis infected with *M. tuberculosis*. The complement system is greatly perturbed in active pulmonary TB. Addition of complement can augment the production of key cytokines and also increase apoptosis induced by *M.tuberculosis*.

(Dr.V.D. Ramanathan, vdramanathan@trcchennai.in)

Evaluation of humoral immune response against ESAT- 6 and ESAT- 6:CFP - 10 polyprotein in diagnosis of tuberculosis

Background:

Development of immunoassays specific for diagnosis of TB requires antigens unique to *M. tuberculosis.* Early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are the two immunodominant antigens encoded by region of difference (RD)-1, which are present only in *M. tuberculosis* complex, but not in BCG. Using CFP-10 alone, we could obtain reasonably good sensitivity in TB cases. This interesting observation provided the impetus for working on its dimeric coprotein ESAT-6 and polyprotein (comprising of ESAT-6 and CFP-10).

Aim:

- To study the ability of species-specific antigen ESAT-6 and ESAT-6:CFP -10 polyprotein to enhance sensitivity, when combined with the species-specific antigen 38kDa

Methods:

Enzyme linked immunosorbent assay (ELISA) for antibody estimation (IgG and IgA) was carried out in the following groups:

Disease groups:

HIV seronegative tuberculosis (n=319):

1. Smear and culture positive patients with pulmonary TB (S+C+) (n=142)
2. Smear negative and culture positive patients (S-C+) with pulmonary TB (n=35)
3. Smear and culture negative, but radiologically diagnosed cases (S-C-) (n=142)

HIV-TB coinfected subjects (n=138):

4. S+C+ (smear and culture positive) (n=77)
5. S-C+ (smear negative and culture positive) (n=43)
6. S-C- (smear negative culture negative) (n=18).

Control groups (n=201):

7. Normal healthy subjects (NHS) (n=126)
8. HIV infected subjects (n=75)

Results:

The levels of IgG and IgA were measured individually and the results were combined for analysis. The cut-off value for each antigen was determined using mean O.D. + 3 SD of NHS.

The results are given in Table 6.2**.** While combining results of both the isotypes the ESAT-6 showed sensitivities of 18%, 24% and 54% and polyprotein showed 6%, 23% and 24% in S+C+, S-C+ and S-C- cases. ESAT-6 and polyprotein both showed more than 95% specificities.

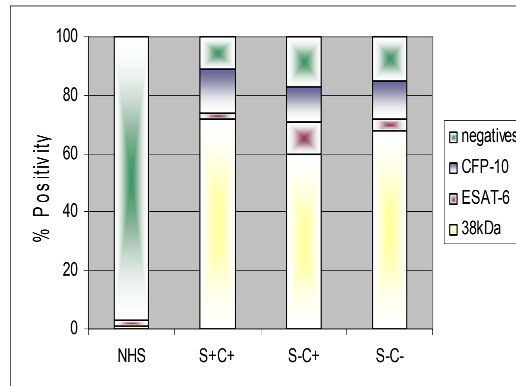
Table 6.2: Antibody profiles of ESAT-6 and Polyprotein

Antigens	Subjects	Number of samples tested	Number of samples detected		
			IgG	IgA	IgG+IgA
ESAT-6	S+C+	113	8(7)	16(14)	20(18)
	S-C+	35	5(14)	11(31)	12(24)
	S-C-	136	29(21)	63(46)	73(54)
	NHS	124	1(1)	2(2)	3(2)
Polyprotein	S+C+	113	7(6)	0(0)	7(6)
	S-C+	35	8(23)	1(2)	8(23)
	S-C-	136	24(18)	21(15)	32(24)
	NHS	124	2(2)	3(2)	4(3)

NHS Normal healthy subjects
S+C+ Smear positive and culture positive PTB patients
S-C+ Smear negative and culture positive PTB patients
S-C- Smear negative and culture negative PTB patients

The combination of 38kDa, CFP-10 and ESAT-6 showed sensitivities of 89%, 83% and 85% in S+C+, S-C+ and S-C- cases with the specificity of 97% (Fig. 6.4).

Fig.6.4: Combination of 38kDa, CFP-10 and ESAT-6



NHS	Normal healthy subjects
S+C+	Smear positive and culture positive PTB patients
S-C+	Smear negative and culture positive PTB patients
S-C-	Smear negative and culture negative PTB patients

Similar analysis of results obtained with the HIV-TB group showed that using ESAT-6 antigen alone or ESAT-6; CFP-10 polyprotein has lower utility in patients infected with HIV.

Conclusion:

Combination of ESAT-6 with 38kDa and CFP-10 improves the sensitivity by 14% in S-C+ cases, where the diagnosis is the most needed.

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Influence of HLA-DRB1 alleles on cytokine response to *M. tuberculosis* and its culture filtrate antigen in pulmonary tuberculosis

Background:

Our earlier studies revealed that HLA-DR antigens can influence humoral and cell mediated immune response to TB. Cytokines play a central role in immune response to *M. tuberculosis* infection. Specific antigen presentation to CD4+ T cells and HLA-DR might modulate cytokine response to *M. tuberculosis* infection.

Aim:

- To study the influence of HLA-DRB1 alleles on live *M. tuberculosis* and its culture filtrate antigen (CFA) induced cytokine response by measuring Th1

(IFN-γ & IL-12p40), Th2 (IL-4 & IL-5), pro-inflammatory (IL-6 & IL-8) and anti-inflammatory (TGF-β & IL-10) cytokines in healthy controls and newly diagnosed PTB patients

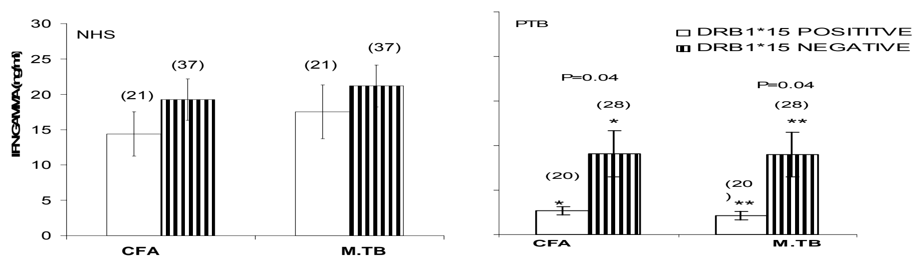
Methods:

Study subjects consisted of 58 NHS and 48 PTB patients. Cytokine levels of IFN-γ, IL-12p40, IL-4, IL-5, IL-6, IL-8, IL-10 and TGF-β in the culture supernatants of PBMC stimulated with *M.tuberculosis* and its CFA, were measured using commercial ELISA kits. HLA-DRB1 genotyping was carried out by DNA based PCR and dot blot hybridization with allele specific oligonucleotide probes and detection by chemiluminescence method.

Results:

Pulmonary TB patients showed significantly increased levels of IL-6, IL-8 and TGF-β and decreased levels of IFN-γ, IL-12p40 and IL-10 in response to live *M.tuberculosis* and its CFA as compared to NHS. Patients with HLA-DRB1*15 allele exhibited a significantly lower IFN-γ response compared to respective allele negative patients (p=0.04) (Fig. 6.5).

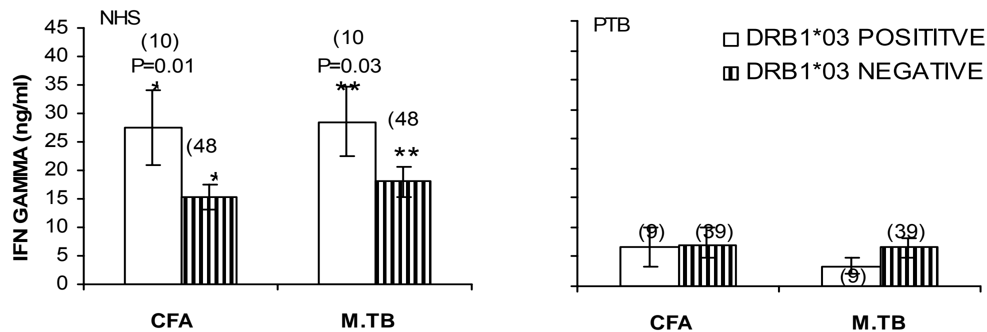
Fig-6.5: CFA and live *M. tuberculosis* induced IFN-γ level in HLA-DRB1*15 positive and negative NHS and PTB patients



An increased IFN-γ response was observed in HLA-DRB1*03 compared to respective allele negative NHS (p=0.03) when stimulated with live *M. tuberculosis* and culture filtrate antigen (Fig. 6.6). An increased level of IL-12p40 was observed in DRB1*10 (p=0.02) and IL-10 in DRB1*12 (p=0.03) positive NHS and an increased level of IL-6 in DRB1*04 (p=0.02) positive PTB patients, compared

to respective allele negative individuals. HLA-DRB1 alleles did not modulate IL-4 and IL-5 response to *M. tuberculosis* and its CFA.

Fig. 6.6: CFA and live *M. tuberculosis* induced IFN-γ level in HLA-DRB1*03 positive and negative NHS and PTB patients



Conclusion:

The study revealed that HLA-DRB1 alleles differentially modulate Th1 cytokines namely IFN-γ and IL-12p40, anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine IL-6 in response to *M. tuberculosis* and its antigens. This suggests that HLA-DRB1 alleles might modulate the immune response to *M. tuberculosis* infection by altering cytokine production.

(PI: Dr. P. Selvaraj; selvarajp@trcchennai.in)

Regulatory role of variant genotypes of vitamin-D receptor gene on cytokine response in pulmonary tuberculosis

Background:

Our earlier studies revealed the immunomodulatory effects of vitamin D_3 on macrophage phagocytosis and lymphocyte functions in TB. Studying the influence of vitamin D_3 on mycobacterial antigen induced cytokine response will help in understanding the basic molecular events associated with pulmonary TB.

Aim:

- To study the regulatory role of vitamin D receptor gene variants on vitamin D_3 modulated Th1 and Th2 cytokine production in response to *M.tuberculosis* antigens in pulmonary TB

Methods:

Peripheral blood mononuclear cells isolated from 60 active PTB patients and 65 NHS were cultured with live *M.tuberculosis* $H_{37}Rv$ and its culture filtrate antigen in the presence and absence of vitamin D_3 and maintained for 72 hours at 37° C and 5% CO2. The cytokines IL-8, IL-6, IFN-γ, IL-12p40, IL-2, IL-10, TGF, IL-4 and IL-5 were estimated in the culture supernatants by commercially available ELISA kits.

Genotyping of vitamin D receptor (VDR) gene was done for *Apa* 1, *Bsm* 1 , *Taq* 1 and *Fok* 1 polymorphisms by PCR-RFLP.

Results:

In normal healthy subjects, vitamin D_3 significantly suppressed IL-12p40 and IFN-γ production in response to CFA and live *M.tuberculosis* in a dose dependent manner with a maximum suppression at 10^{-7}M concentration ($p<0.0001$). In patients, a significant dose dependent decrease in IL-12p40 levels in response to CFA (10^{-7}M, $p<0.0001$) and live *M. tuberculosis* (10^{-7}M, $p <0.0001$) (Fig. 6.7) and a significant decrease in IFN-γ production in response to CFA (10^{-7}M, $p=0.01$) was observed. In healthy controls, the IL-2 levels were increased upon vitamin D_3 addition in CFA stimulated cultures, but no effect was seen in patients (Figure 6.8 a&b). Addition of vitamin D_3 showed an increased trend for IL-4 and a decreasing trend for IL-5 levels.

Fig. 6.7 – Effect of Vitamin D_3 on IL-12 p40 levels in NHS and PTB

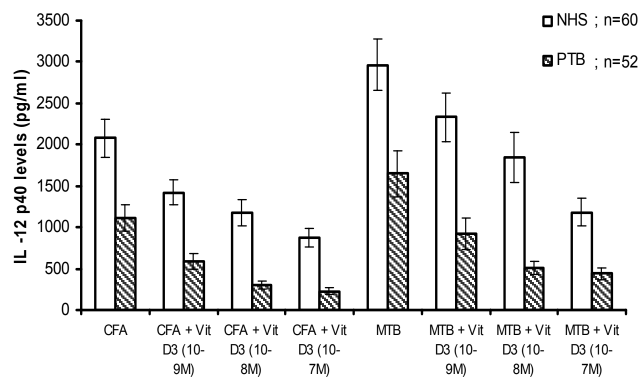
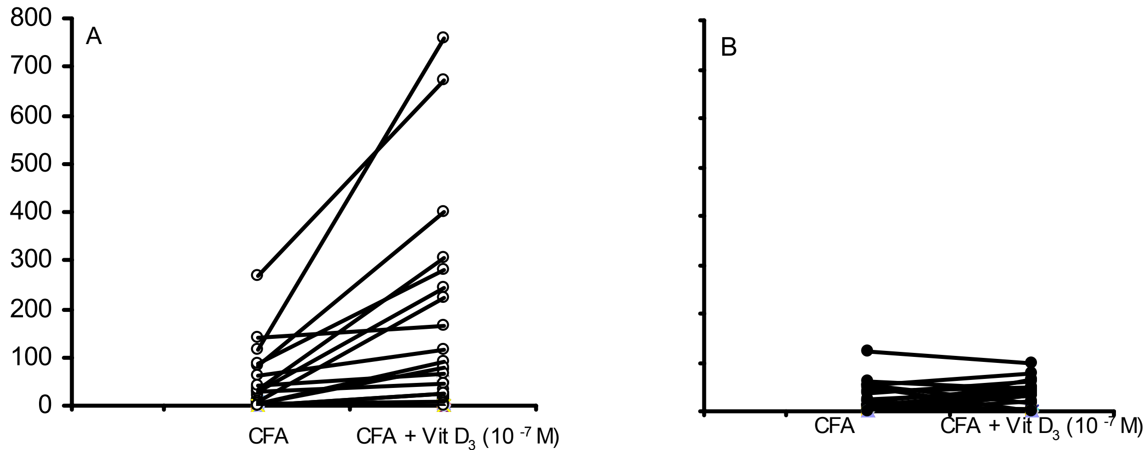


Fig.6.8a&b: a) Effect of vitamin D_3 on CFA induced IL-2 in NHS
b) Effect of vitamin D_3 on CFA induced IL-2 PTB



The cytokine levels were further correlated with the vitamin D receptor genotypes to study the regulatory role of VDR gene variants on vitamin D_3 modulated cytokine production. In CFA stimulated cultures of NHS, the IFN-γ level was increased in 'tt' genotype when compared to TT genotype. The IL-10 production was significantly increased in *M.tuberculosis* and vitamin D_3 treated cultures (1×10^{-9} M; p=0.05, 1×10^{-8} M and 1×10^{-7} M; p=0.01) in TT genotype when compared to 'tt' genotype in NHS. In PTB, the IL-10 levels were slightly increased in patients with 'tt' genotype, but it was not statistically significant.

Conclusions:

The present study suggests that vitamin D_3 exerts differential effects on cytokine production in TB with a prominent suppressive effect on IL-12p40 and IFN-γ and that the variant VDR genotypes regulate the vitamin D_3 modulated cytokine response.

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Human leucocyte antigen (HLA) and non-HLA gene polymorphism studies in HIV and HIV-TB patients

Background:

In developing nations, HIV-1 infection has increased the burden of TB especially in populations where the prevalence of TB infection is high among young adults. The importance of host genetic factors (HLA and non-HLA) on susceptibility or resistance to HIV infection and the variability in disease progression towards AIDS has been emphasized by many studies.

Aim:

- To find out whether polymorphisms in Human Leucocyte Antigens (HLA) –A, –B, -DR and –DQ antigens and non-HLA genes (Mannose binding lectin gene) are associated with susceptibility or resistance to HIV and HIV-TB

Methods:

The study subjects include 151 HIV positive TB negative patients (HIV+TB-), 117 HIV positive TB positive patients (HIV+TB+), 162 HIV negative TB positive patients (HIV-TB+) and 186 healthy controls. HLA –A, –B antigens were typed using microlymphocytotoxicity assay and -DR and –DQ typing was done by PCR with locus specific primers followed by hybridization with allele specific oligonucleotide probes (ASOP) and detection by chemiluminescence method. Mannose binding lectin-2 (MBL-2) structural genotypes (54, 57 and 52) were identified using PCR-ASOP. Promoter genotypes (-221 X/Y) were identified using PCR with allele specific primers. MBL levels from plasma were estimated using an MBL oligomer ELISA kit.

Results:

Highly decreased frequency of HLA –A11 was observed in HIV patients with and without TB compared to controls (Controls vs Total HIV: Pc=0.00044; Controls vs HIV+TB-: p=0.021; Controls vs HIV+TB+: Pc=0.0001). Significantly increased frequency of HLA –B40 was noticed in HIV patients with and without TB compared to controls (Controls vs Total HIV: Pc=0.014; Controls vs HIV+TB- p=0.011; Controls vs HIV+TB+: Pc=0.017). Increased frequency of HLA –DR2

was noticed in HIV patients with and without TB compared to controls (Controls vs Total HIV: Pc=0.024; Controls vs HIV+TB- : Pc=0.024; Controls vs HIV+TB+ p=0.036). Significant increase of HLA –DR2 was also observed in HIV-TB+ as compared to controls (Controls vs HIV-TB+ Pc=0.043) (Table 6.3). Data on DQB1 typing is being analysed.

Table 6.3: Percent frequencies of selected HLA antigens among healthy controls, HIV patients without and with TB and HIV negative patients with TB

HLA antigens	Healthy controls (n=186) %F	Total HIV infected patients (n=268) %F	HIV+TB- (n=151) %F	HIV+ TB+ (n=117) %F	HIV-TB+ (n=162) (%F)
A11	28.5(53)	12.7(34)	17.2(26)	6.8(8)	24.7(40)
B40	21.0(39)	36.2(97)	33.8(51)	39.3(46)	31.5(51)
DR2	34.3(47)	49.4(124)	53.2(74)	48.5(50)	51.9(80)

For DR, n=137 for controls, n=139 for HIV+TB-, n=103 for HIV+TB+, n=154 for HIV-TB+.
Numbers in the parentheses represent individuals positive for that genotype
n= number of subjects studied, F= antigen frequency.

Plasma MBL levels were significantly higher in HIV+TB+ patients compared to HIV+TB- patients. A significantly increased frequency of YA/YA diplotype (associated with high MBL levels) was observed among HIV patients with TB compared to controls (p=0.0108). A significantly increased frequency of YO/YO diplotype (associated with low MBL levels) was observed among HIV negative TB patients than in controls (p=0.036) (Table 6.4).

Table 6.4: Percent frequencies of selected MBL diplotypes among healthy controls, HIV patients without and with TB and HIV negative patients with TB

Diplotypes	Healthy controls (n=146) %F	HIV+TB- (n=151) %F	HIV+ TB+ (n=112) %F	HIV-TB+ (n=146) (%F)
YA/YA	19.2 (28)	23.2 (35)	33.9 (38)	26.7 (39)
YO/YO	4.8 (7)	8.6 (13)	6.2 (7)	12.3 (18)

Numbers in the parentheses represent individuals positive for that genotype
n= number of subjects studied, F= Diplotype frequency

Conclusion:

The study suggests that HLA –A11 may be associated with resistance to HIV infection and HIV with active TB, while HLA –B40 and HLA –DR2 may be associated with susceptibility to HIV and HIV with active TB. This finding may have implications for vaccine design and to develop effective prophylactic strategies against HIV and TB. High MBL levels and diplotypes associated with increased MBL levels might have a predisposing effect on the development of TB in HIV infected individuals and diplotypes associated with low MBL levels may be a risk factor for development of TB in HIV negative individuals.

(PI: Dr. P. Selvaraj; : selvarajp@trcchennai.in Funding: ICMR Task Force)

Human monocyte and macrophage apoptosis induced by *M.tuberculosis* strains and its implication on cell mediated immune response

Background:

Apoptosis is an orchestrated suicide program, which enables macrophages to remove invading pathogens without inducing an inflammatory response. The regulation of programmed cell death is a complex process involving a myriad of proteins within the cell. In TB, the reduction in bacillary load is associated with apoptotic death of infected macrophages at the site of infection. The ability of *M. tuberculosis* to survive within the macrophage for an extended period is its

greatest success. Hence it is important to understand the apoptotic signals and survival strategies of the *M. tuberculosis* to offset cell death for pathogenesis. Our previous findings suggested that two prevalent clinical strains of *M. tuberculosis* acted differentially in skewing the Th response. Taking this as a cue we further probed to study how these strains modulated the macrophage apoptosis and the mechanism of modulation was also studied.

Aim:

- To delineate the molecular mechanisms of apoptosis and survival strategies in infected THP-1 macrophages with different strains of *M. tuberculosis*

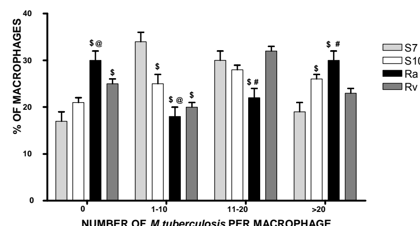
Methods:

Phorbol myristate acetate (PMA) differentiated THP-1 macrophages were infected with two clinical isolates (S7 and S10) and two laboratory strains ($H_{37}Rv$ and $H_{37}Ra$) of *M. tuberculosis* for various time points. Apoptosis was studied using annexin –V FITC labeling and survival of *M. tuberculosis* strains within the cells was detected by fluorescence microscopy. The expression of two components system *prrA–prrB* and *mprA-mprB* in *M. tuberculosis* strain was done with reverse transcriptase - PCR (RT-PCR). The cell lysates were subjected to ECL immunoblotting for various cell death and cell survival molecules.

Results:

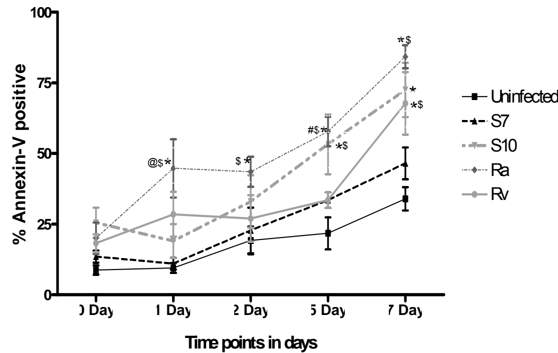
The percentage phagocytosis did not show any strain specific association with differentiated THP-1 cells. But in phagocytic index, the clinical strains showed low dose of infection in 1-10 bacilli category thereby exerted less burden on the cells (Fig 6.9 & 6.10).

Fig 6.9: Phagocytosis of various *M. tuberculosis* strains by THP-1 cells



Each bar represents the mean value of five different experiments. The statistical significance is shown as $ compared to S7, @ compared to S10, # compared to H37Rv when P<0.05.

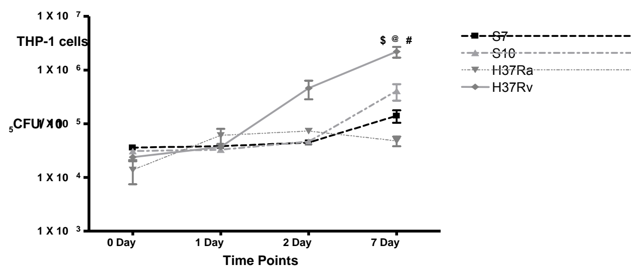
Fig. 6.10: Apoptosis induced by various *M. tuberculosis* strains



Above values are mean ± SEM obtained from four different experiments. The statistical significance is shown as * compared to uninfected, $ compared to S7, @ compared to S10, # compared to H37Rv when P<0.05.

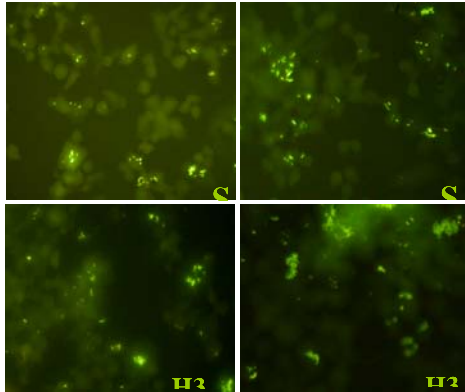
The induction of apoptosis was strain dependent (Fig 6.11 & 6.12). A delayed onset in expression of the two systems *prrA–prrB* and *mprA-mprB* in clinical strains compared to the laboratory strains was observed. At later time points of 7 and 14 days, a differential activation of these molecules in clinical isolates (S7 and S10) compared to the laboratory strains (H37Rv and Ra) was observed. Importantly, on day 7, marked upregulation of XIAP molecule was observed in S7 infected macrophages. The differential kinetics of these molecules may inhibit their initial multiplication and growth but support for longer survival in the macrophages.

Fig.6.11: Intracellular growth of various *M.tuberculosis* strains



Above values are mean ± SEM obtained from four different experiments. The statistical significance is shown as * compared to uninfected, $ compared to S7, @ compared to S10, # compared to H37Rv when P<0.05.

Fig. 6.12



Photomicrographs show THP-1 cells infected with various strains of *M. tuberculosis* (S7, S10, H37Ra and H37Rv). The infected cells were stained with auramine-KMnO4 after 3 hours of phagocytosis. The picture was taken with a fluorescence microscope (U-ND25-2; Olympus, Tokyo, Japan) at high power magnification (X400). The bacilli are bright yellow and the THP-1 cells are green.

Conclusion:

The results demonstrated differential apoptosis of *M. tuberculosis* infected THP-1 macrophages at later time points. By virtue of these pathways, virulent *M. tuberculosis* able to up-regulate the macrophage survival signal, thereby creating an environment favorable for the survival of the pathogen.

(PI: Dr. Sulochana Das; sulochanad@trcchennai.in.)

Molecular epidemiology of *Mycobacterium tuberculosis*

Background:

Mycobacterium tuberculosis has a global population structure, and there is increasing evidence that strain genetic differences have important phenotypic consequences. Early studies reported that strains from south India were less virulent in guinea pigs than strains from other regions.

Aim:

- To determine the population structure of *M. tuberculosis* in south India and identify potential genomic correlates of attenuation and virulence, using isolates from a population-based molecular epidemiologic study

Results:

To estimate the frequency of the six newly identified large sequence polymorphisms (LSPs) (Table 6.5) among the isolates with RD239 deleted, and to see whether the new LSPs were associated with different strain phenotypes,

100 isolates were chosen from the largest spoligotype clusters and screened by PCR for the six LSPs (RD768-RD773).

Table 6.5: Description of six new LSPs or genomic deletions observed in the clinical isolates from south India

Isolate	Name*	Coordinates start	Coordinates stop	Primer sequence (5' …… 3')	Size† (bp)	Putative function of deleted genes and base pairs
M394	RD768	70289	72023	GGG GCG GCT GTT GGA CCC GCA TAT CCT	1735	Rv0064 Transmembrane protein
M357	RD769	1957030	1959686	GAC AGC AAC CGC GAC GCC CGG AAT C	2657	Rv1730c Pencillin binding protein
						Rv1731 (*gabD2*) Succinate-semialdehyde dehydrogenase
				CCC GCC CTC GTC GTC ACC TTC ATC TGT AA		(NADP+) dependent (SSDH) *gabD2*
						Rv1732c Conserved hypothetical protein
M395	RD770	2196902	2200337	CCG GTG ACC GTC GTG GTG AGC ACC A	3436	Rv1946 (*lppG*) Possible lipoprotein
						Rv1947 Hypothetical protein
				CCA GGA CGG AGG TCA CAG TTG CGG GGT		Rv1948 Hypothetical protein
M118	RD771	89500	90450	CCG GGC GCG CGA ACA TGG ACT GC	951	Rv0081 Probable transcriptional regulatory protein
						Rv0082 Probable oxidoreductase
				GGC TCG GCG CCT CCG GGT GG		Rv0083 Probable oxidoreductase
M165	RD772	30669	34074	GCC ATC GCG GAG GCG GAA GCA GCT CT	3406	Rv0027 Conserved hypothetical protein
						Rv0028 Conserved hypothetical protein
				TTT GCC CGG CCT AGC GGT TGC CCA TC		Rv0029 Conserved hypothetical protein
						Rv0030 Conserved hypothetical protein
						Rv0031 Possible remnant of a transposase
M461	RD773	3434523	3441337	CGG CCC TGA CGG TGG CAA TCT GGA TC	6815	Rv3071-Rv3076 Conserved hypothetical
						Rv3077 Possible hydrolase
				GAG CAG GGT CGC CAG CCA GTT GCC		Rv3078 (*hab*) Probable hydroxylaminobenzene mutase
						Hab
						Rv3079c Conserved hypothetical protein
						Rv3080(*pknK*)Probable serine/threonine-protein kinase
						transcriptional regulatory protein *pknK* (protein kinase k)

bp = base pairs
* Name assigned to the specific genomic deletion
† Size of genomic deletion (bp) = (stop coordinates – start coordinates) + 1

However, RD769 and RD771 were each detected in only two of the 100 screened isolates, and RD768, RD770, RD772 and RD773 were not deleted in any of the 100 isolates. Because of the low frequency of these LSPs, we conclude that these genomic alterations are unlikely to account for the low virulence of south Indian strains in animal models.

Conclusion:

Genomic deletion analysis of 1,215 TB patient isolates from south India revealed that 85.2% belonged to the ancestral lineage of *M. tuberculosis*. We identified six new genomic regions within this lineage that were variably deleted. Our findings emphasize the need to consider global strain variation during TB product development.

(PI: Dr Sujatha Narayanan, sujathan@trcchennai.in; Funding: WHO-USAID, Collaborating Institute Stanford University)

Studies in Progress:

The role of complement in the interaction of *M. tuberculosis* with human macrophages

Background:

The complement system, an important component of innate immunity is a potent mediator of inflammation and at the same time plays a pivotal role in modulating the adaptive immune response also. The initial interaction between the complement system, the macrophage and *M. tuberculosis* is an important first step in the pathogenesis of TB and is mediated by specific macrophage receptors and ligands present on the surface of *M. tuberculosis*. Although it is known that *M. tuberculosis* replicates within the host macrophages, the mechanism by which it evades being killed by macrophages remains poorly understood. Since mycobacteria interact with the complement system initially and antimycobacterial antibodies are known to be present in endemic populations, it was considered important to investigate whether antibodies could modulate complement activation and determine the interaction of *M. tuberculosis* with the macrophage.

Aim:

- To study whether antibodies could modulate complement mediated interaction of *M. tuberculosis* with the macrophages

Methods:

Mycobacterium tuberculosis will be treated with complement (classical or alternative pathway) in the presence and absence of IgM or IgG antibodies against *M. tuberculosis.* The following will then be determined:

(i) Investigate the receptor mediated entry of *M. tuberculosis* into the macrophages.
(ii) Intracellular viability of the tubercle bacilli.
(iii) Release of free radicals and different cytokines from macrophages after interaction with *M.tuberculosis.*

Results:

Mycobacterium tuberculosis was pre treated with buffer or antibody and reacted with either the classical or the alternative pathway. This was then added to PBMCs and the expression of complement receptors and the production of cytokines are being evaluated. Of the projected 10 volunteers in whom this is to be evaluated, so far PBMC from five have been done. Preliminary results indicate that while phagocytosis is increased by the pre opsonization with complement, it is not reflected in the production of cytokines such as IL-2 or IFN-γ.

(Dr.V.D. Ramanathan, vdramanathan@trcchennai.in)

Complement activation by gene-disrupted *M. tuberculosis*

Background:

Complement system plays an important role in the opsonization and phagocytosis of mycobacteria. The alternative pathway of complement activation has the ability to recognize a variety of pathogens independent of antibody. However, both classical and alternative pathways are required for optimal phagocytosis of bacteria. Many studies have documented the complement activation potential of various mycobacterial strains and their cellular components. A number of gene-disrupted strains of *M.tuberculosis* are now available and differences in the pathogenic potential of these strains compared to the wild type strain are being delineated now. In view of the importance of the the

innate immune responses in modulating the host-parasite interactions, it is important to investigate the relationship between the complement system and genetically modified strains of *M.tuberculosis*.

Aims:

- To assess complement activation at the level of C3, C4 and factor B by the following gene-disrupted *M. tuberculosis* strains: MptpA, MptpB, VirS, DKO, DevR, complemented strains of all these except DKO and their respective wild strains, *M. tuberculosis* Erdman and H37Rv strains

- To assess the effect of the above mentioned strains of *M.tuberculosis* to modulate the expression of complement receptors on PBMCs and release of various cytokines from them

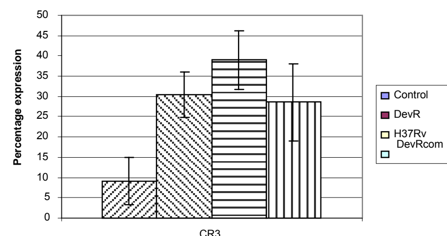
Methods:

All the above strains are grown in Middlebrook 7H9 broth for 3-4 weeks at 37°C. Complement activation at the level of C3, C4 and factor B by *M.tuberculosis* will be assessed using solid phase ELISA. Complement receptor expression will be evaluated using flow cytometry and the quantitation of various cytokines using ELISA.

Results:

Whole blood cells were treated with wild type, gene-disrupted and complement strains of *M.tuberculosis.* The expression of complement receptors CR1 to CR4 using flow cytometry and the production of cytokines were assessed using bead assay by flow cytometry. Preliminary results so far from 4 individuals indicate that the mutant Dev-R induces a lesser expression of CR3 on CD14 positive cells (Figure 6.13).

Fig. 6.13: Expression of CR3 on CD14 cells



(PI : Dr.V.D. Ramanathan, vdramanathan@trcchennai.in)

Identification of immunoreactive T-cell antigens of *M.tuberculosis* through proteomic techniques

Background:

Even though effective chemotherapy is available for treatment of TB, there are practical difficulties in ensuring the desired high cure rate, due to many factors. Immuno-prophylactic measures using vaccines is an alternative approach for control.

In the previous year's Annual Report (2005-2006), a systematic approach to test the antigens purified by two-dimensional (2-D) preparative separations, in human subjects has been described. Results of *in vitro* assays and characterization of the purified antigenic fractions are presented in this report.

Aim:

- To identify a set of immunologically relevant T-cell antigens and evaluate the response to these antigens in patients with TB and controls

Methods:

The study subjects are as follows:

1. Apparently healthy household contacts (HHC) of sputum positive pulmonary TB living in the same household. Tuberculosis was ruled out in this group during the time of blood collection and hence considered "Protected".
2. Newly diagnosed adult pulmonary TB cases. They form the "susceptible" group.

The methods followed are as follows:

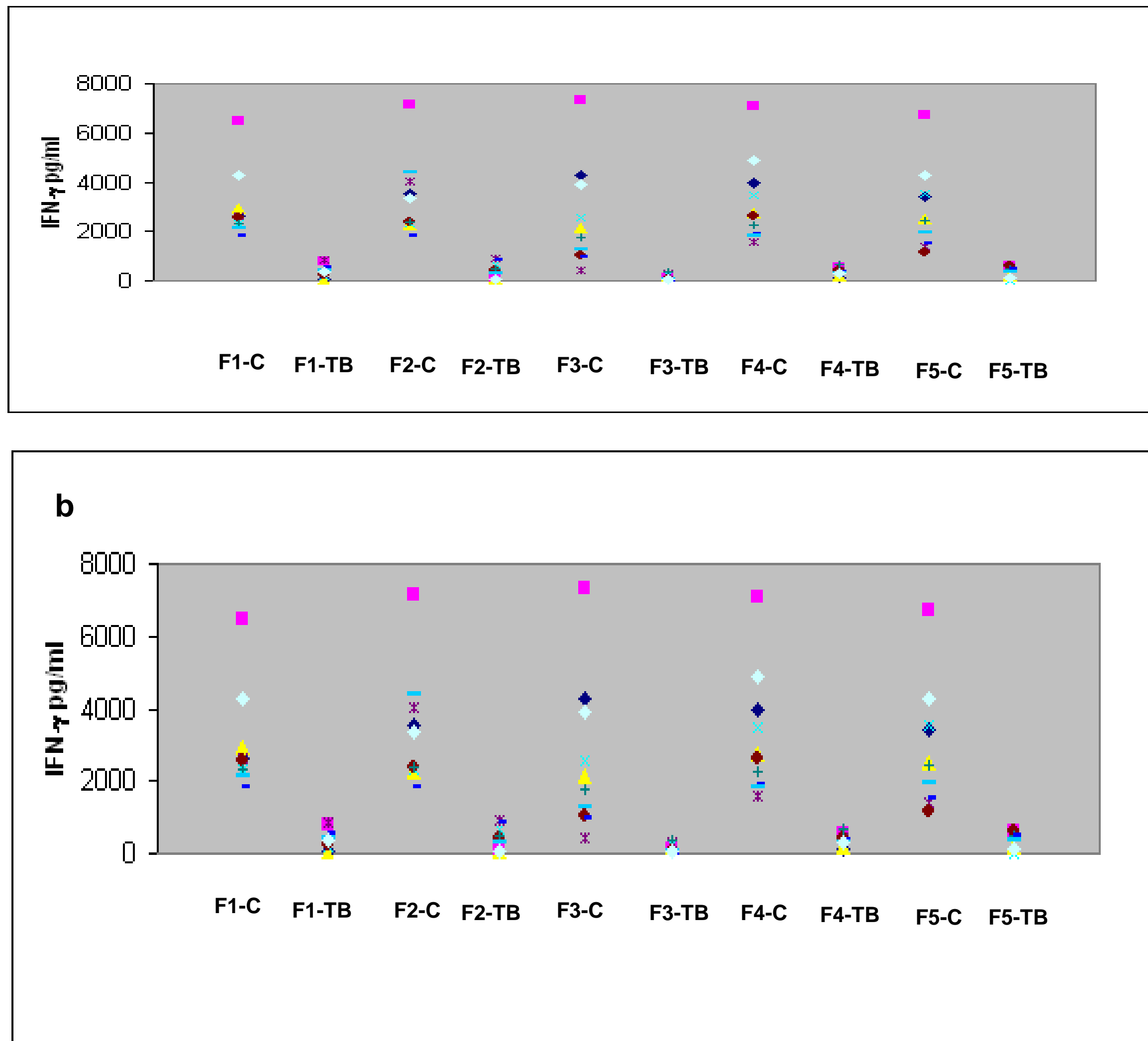
1. Two dimensional Preparatory separation of antigenic fractions
2. Proliferative response and IFN-γ response were studied using purified antigenic fractions.

Results:

Using the preparatory 2-dimensional approach, the *M. tuberculosis* secreted proteins have been separated into 600 fractions. Of these, fractions having at least 50 μg or more were selected for further testing (347 fractions). We have standardized a whole blood (1:10 dilution) assay, for proliferation and IFN-γ secretion.

Interferon-γ secretion pattern of 10 HHC and 10 TB subjects to 10 purified antigenic fractions (F1 to F10) are presented in Fig. 6.14 a & b. Out of the 10 fractions, none of them induced IFN-γ response in TB patients.

Fig. 6.14a & b: IFN-γ Response to highly recognized antigenic fractions



C-Contacts; F1-F10-antigenic fraction; TB-Tuberculosis

The rest of the fractions could be classified as those inducing response in 9 HHC and 0 TB; 8 HHC and 0 TB; 9 HHC and 1 TB; 8 HHC and 1 TB etc.

All fractions have been subjected to tandem mass spectrometry, using online multidimensional nano HPLC and electrospray ionization (ESI) – ion trap technology, with the kind help of Dr. John Belisle, Colorado State University, USA. Mass spectrum of each fraction was analyzed with Bioworks 3.3 software which uses the SEQUEST protein search algorithm and identifies the protein. Protein identity of each

fraction was further confirmed by analyzing in Scaffold Protein Prophet software, which uses SEQUEST and MASCOT protein search algorithm and results are expressed as percentage of probability of a particular protein present in the sample.
In addition, each fraction was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. Each fraction showed 1 or a maximum of 2 bands. The molecular weights and identity of the protein are being analyzed.

(PI: Dr. Alamelu Raja, alamelur@trcchennai.in)

Cytotoxic cell response in *M. tuberculosis* infection

Background:

Cytotoxic T lymphocytes are required for protective immunity against intracellular pathogens such as *M. tuberculosis*, pathogens known to escape from phagocytic vacuoles into the cytoplasm of infected host cells. Cytotoxic T cells are needed to release bacteria from the ineffective macrophages so that they can be phagocytosed by fresh, fully activated monocytes or macrophages.

Aim:

- To analyse the cytotoxic cell response in TB

Methods:

The study subjects are the two groups as described above, "susceptible" and "protected". Response to whole *M. tuberculosis* $H_{37}Rv$, purified antigens such as ESAT-6 and CFP-10 and overlapping 20-mer peptides of the 2 purified antigens are being studied. Lymphoproliferation of CD4 and CD8 cells [Anti-bromodeoxyuridine (Brdu) anti BrdU labeling, flow cytometry)] and IFN-γ secretion (ELISA) to these antigens are also carried out.

Results:

Initially, cytokine response to whole *M. tuberculosis* $H_{37}Rv$ was studied among patients and normal subjects. It was observed that there was no difference between unstimulated (control) and $H_{37}Rv$ stimulated cells in cytokine secretion. Considering the two groups, it was observed that there was a decrease in cytokine production in

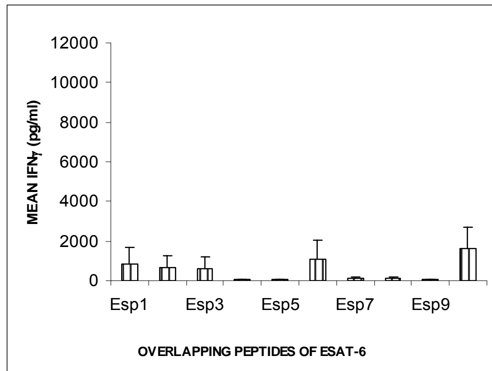
TB patients when compared to normals. The response of both CD4 and CD8 T cells was similar.

Cytolytic molecule response was also studied in these two groups to *M. tuberculosis*. There was no difference among stimulated and unstimulated cultures to various cytolytic molecule secretions. An increase in Granzyme A and B levels was observed in patients than HHC. Cytotoxic T (CD8) cells were found to produce more Granzyme A and B when compared to helper cells (CD4).

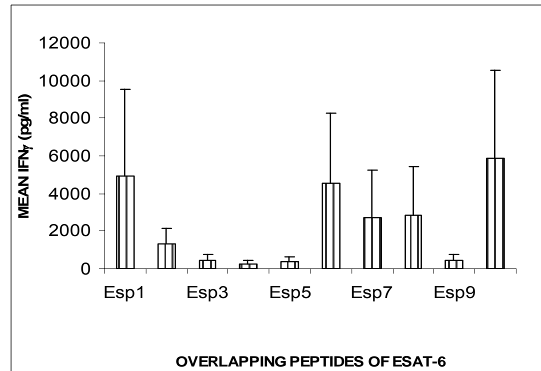
IFN-γ secretion in response to each of the overlapping 20-mer peptides of ESAT-6 and CFP-10 was measured. It was observed that ESAT-6 peptides 1, 6, 7 and 8 were immunogenic inducing IFN-γ. Peptides 7 and 8 were found to stimulate only lymphocytes from HHC and not TB (Fig. 6.15a - d).

Fig 6.15 a, b, c, d: Mean IFN-γ response among patients and contacts to overlapping peptides of ESAT-6 and CFP-10 antigen

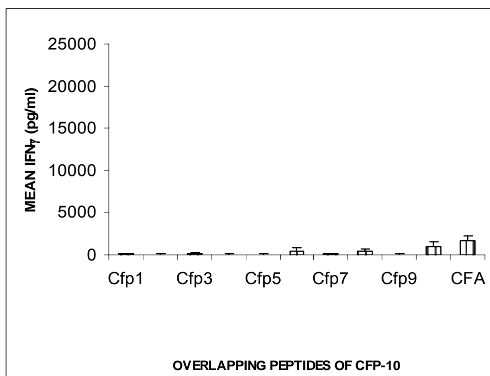
a. Patient



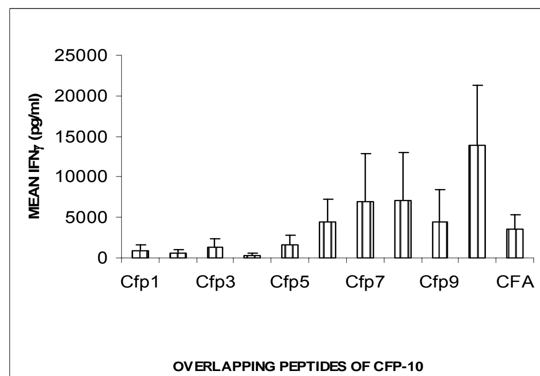
b. Contact



c. Patient

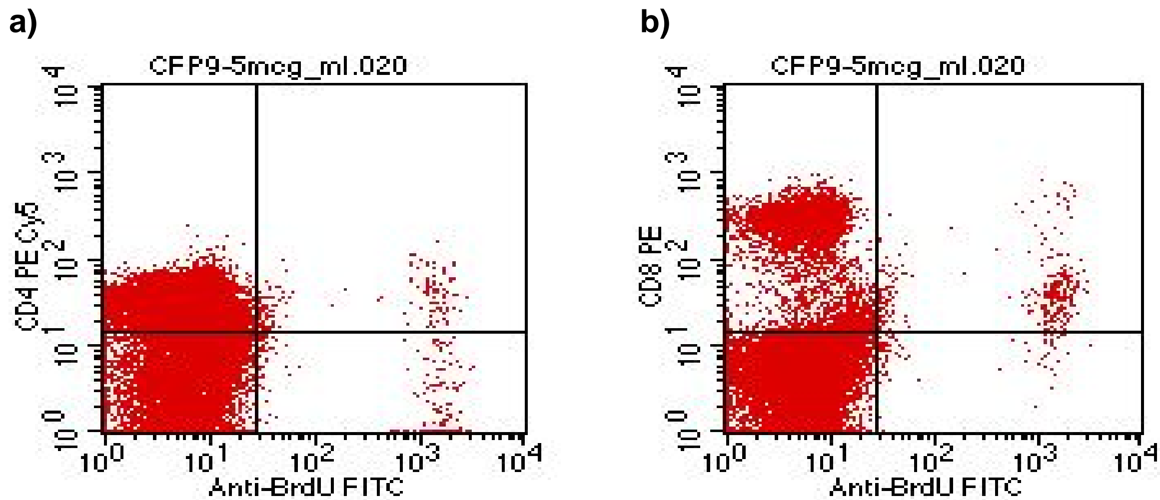


d. Contact



Similarly, CFP-10 peptides 7, 8 and 9 induced more response in HHC than in TB. It was observed that the peptides stimulated both CD4 and CD8 cells and proliferation to peptides [anti-Brdu labeling] was more marked in CD8 cells than CD4 T cells (Fig. 6.16a & b). .

Fig. 6.16a & b: Representative dot plot showing CFP10 - peptide 9 (5 μg/ml) responding CD4 & CD8 population by BrdU proliferation assay



(PI: Dr. Alamelu Raja, alamelur@trcchennai.in)

Innate immunity in HIV infection:

Background:

Natural Killer (NK) cells are innate effector cells of the immune system, which exert a prompt cytolytic activity against infected and tumor cells without prior stimulation. NK cells are active in the earliest stages of the host defense, display broad specificity and rapid activation.

Aim:

- To demonstrate NK cell mediated innate immune response in HIV-TB. Various innate immune parameters mediated through unstimulated and stimulated NK cells will be studied

Methods:

The study subjects consisted of 12 HIV seronegative TB patients and 8 NHS.

The methods to be followed are as follows:

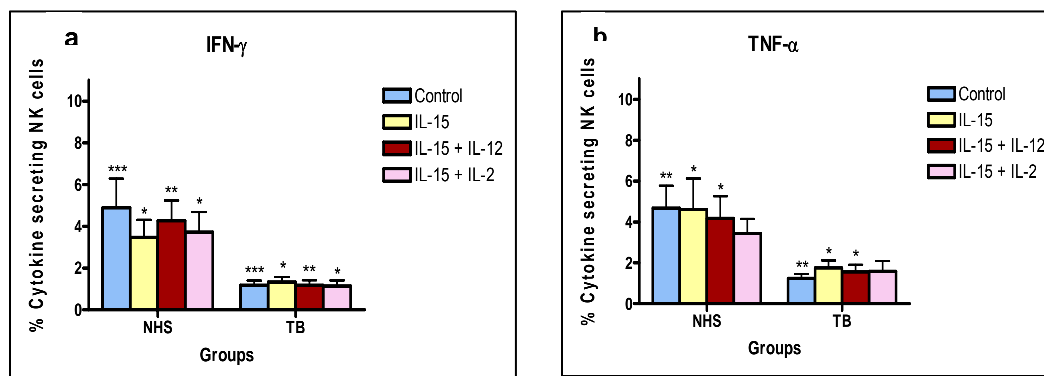
In vitro response of NK cells to *M. tuberculosis* $H_{37}Rv$ will be studied using intracellular cytokine secretion (IL-2, IL-4, IFN-γ and TNF-α). Cytotoxic molecules (Granzyme A, Granzyme B and Perforin) positive cells will be determined by flow cytometry. Response of NK cells after *in vitro* stimulation with cytokines (IL-15, IL-15+IL-12, IL-15+IL-2). Cytotoxic response of NK cells against tumor cell lines (K562) will be studied using flow cytometry.

Results:

Using *M. tuberculosis* $H_{37}Rv$ stimulation *in vitro*, both Th1 and Th2 cytokines, IL-2, TNF-α, IFN-γ and IL-4 were measured intracellularly, by flow cytometry. In both the groups, the percentage of IL-2 secreting NK cells was not altered in the presence or absence of *M. tuberculsis* $H_{37}Rv$ stimuli. Similar results were observed with other cytokines, IL-4, IFN-γ, and TNFα. The percentage of cytokine positive NK cells (>5%) was higher when compared with adaptive cells CD4 and CD8 (<2%).

It was observed that all the 4 cytokines measured *ex-vivo*, were significantly ($p<0.01$ to 0.001) reduced in NK cells of TB patients, as compared to that of NHS. Since it is known that dysregulation of cytokines play a major role in infectious diseases, the effect of *in vitro* supplementation of cytokines was studied. When the cells were stimulated *in vitro* with stimulants like IL-12+IL-15, the situation did not improve (Fig. 6.17 a & b).

Fig 6.17 a & b: Natural Killer cell response against *in vitro* stimulation with cytokines



NHS Normal healthy subjects
Pulmonary TB patients

The NK cells from both TB and NHS groups constitutively expressed cytolytic molecules such as perforin, Granzyme A and Granzyme B. The percentage of NK cells expressing cytolytic molecules did not change significantly when stimulated with H37Rv. The results were similar between TB and NHS groups. In general, cytolytic molecule positive NK cell levels were significantly greater than CD4 or CD8 cells (Table 6.6).

Table 6.6: Cytolytic response of lymphocytes against *M. tuberculosis* infection

Cytolytic molecules	**NK**		**CD4**		**CD8**	
	Unstimulated	H37Rv	Unstimulated	H37Rv	Unstimulated	H37Rv
Perforin	11.74	10.02	1.14	0.87	5.76	2.26
GranzymeA	37.91	29.23	5.35	3.28	26.82	18.97
GranzymeB	22.43	20.08	2.47	2.39	11.02	12.49

Values are given as Mean percentage

(PI: Dr. Alamelu Raja, alamelur@trcchennai.in.)

Interferon gamma assay for latent TB in HIV infection

Background:

HIV infection is a risk factor for rapid progression of a recently acquired TB infection and for re-activation of latent TB infection (LTBI). Because of the associated higher risk of mortality, tests that detect *M. tuberculosis* infection and disease at early stages, are needed to initiate chemoprophylaxis / therapy.

Aim:

- To assess the role of Interferon gamma (IFN-γ), Interleukin-4 and Interleukin-4δ2 in the diagnosis of latent TB

Methods:

The study subjects are as follows:

1. Healthy household contacts (HHC), with high risk for LTBI (n = 200)
2. Healthy controls (HC), with low risk for LTBI (n = 200)
3. HIV +ve (n = 200)
4. TB +ve (n = 100)
5. HIV-TB (n = 100)

Skin test was performed and read as per standard procedures, using two TU of PPD RT23 (Statens Serum Institute, Denmark) and reading was taken 48-72 hrs post testing. Whole blood Interferon gamma releasing assay was done by using Quantiferon TB Gold kit (Cellestis, Victoria, Australia) as per the manufacturer's instructions.

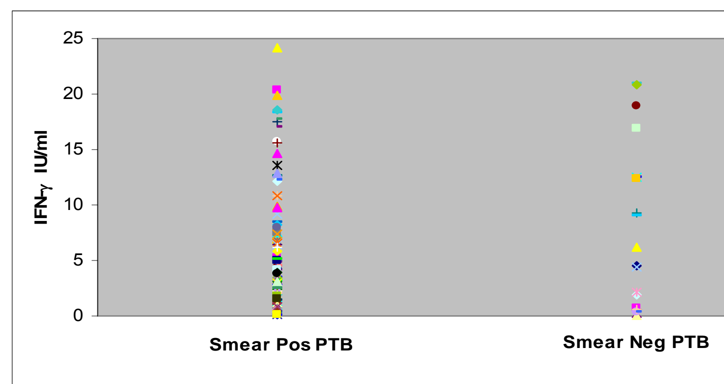
Results:

Sensitivity of interferon gamma release assay in active TB patients

Among the total number of 113 TB subjects, 81 were male and 32 were female with the median age average of 37 (Range 10-65). All the study subjects were diagnosed based on clinical, radiological and microbiological parameters.

Among the total study subjects, maximum numbers have been recruited in TB cases. The levels of IFN-γ have been measured (Fig. 6.18) 0.35 IU/ml was set as cut-off value as described by the manufacturer. IGRA showed sensitivity of 90% in both smear positive as well as negative cases. The percentage of positivity did not vary between smear positive and negative cases.

Fig.6.18: Levels of IFN-γ in Pulmonary TB patients



The horizontal line shows the kit cut-off point 0.35 IU/ml.

PI: Dr. Alamelu Raja, alamelur@trcchennai.in Funding: R03 Grant, NIH)

Human leucocyte antigen -DP (HLA-DP) and vitamin-D receptor (VDR) gene polymorphism studies in HIV and HIV-TB patients

Background:

Our recent studies revealed that HLA –A11 was associated with resistance to HIV and HIV with active TB (HIV-TB), while HLA –B40 and –DR2 were associated with susceptibility to HIV and HIV-TB. Moreover polymorphisms in VDR gene variants are shown to be associatied with differential susceptibility or resistance to TB.

Aim:

- To find out whether HLA –DP and VDR gene variants are associated with susceptibility or resistance to HIV and HIV-TB

Methods:

The study subjects include 151 HIV positive TB negative patients (HIV+TB-), 117 HIV positive TB positive patients (HIV+TB+), 150 HIV negative TB positive patients (HIV-TB+) and 150 healthy controls. VDR-Taq1 polymorphism was studied using PCR with sequence specific primers followed by digestion with Taq1 restriction enzyme using RFLP method.

Results:

An increased frequency of Tt genotype was observed among HIV patients without TB as compared to controls (p=0.02) while a decreased frequency of 'tt' genotype was seen in HIV patients without TB compared to HIV patients with TB (p=0.02) (Table 6.7). Studies on other polymorphisms in VDR gene (CDX2, A1012G, Fok1, Bsm1 and Apa1) are in progress. Studies on HLA –DP typing are in progress.

Table 6.7: Percent genotype frequencies (%GF) of VDR Taq1 polymorphism among healthy controls, HIV patients without and with TB

VDR Taq 1 genotypes	Healthy controls (n=132) %GF	HIV+ TB- (n=148) %GF	HIV+ TB+ (n=114) %GF
TT	47 (62)	36.5 (54)	36.8 (42)
Tt	40.9 (54)	54.05 (80)	43.7 (50)
tt	12 (16)	9.5 (14)	19.3 (22)

Numbers in the parentheses represent individual positive for that genotype
n= number of subjects studied

(PI: Dr.P.Selvaraj; selvarajp@trcchennai.in)

Role of variant genotypes of vitamin-D receptor gene on plasma vitamin D_3, vitamin D receptor expression and intracellular cytokine positive cells in pulmonary tuberculosis

Background:

Our earlier studies revealed that VDR gene variants regulate macrophage phagocytosis, lymphocyte function and various cytokine responses to *M. tuberculosis* antigens in normal healthy subjects and pulmonary TB patients. Studying the role of variant genotypes of VDR gene on plasma vitamin D_3 level, VDR expression and intracellular cytokine positive cells will explore the basic molecular events associated with vitamin D_3 and immunity to TB.

Aim:

- To study the regulatory role of variant genotypes of VDR gene on plasma vitamin D_3, VDR expression and intracellular cytokine positive cells in pulmonary TB

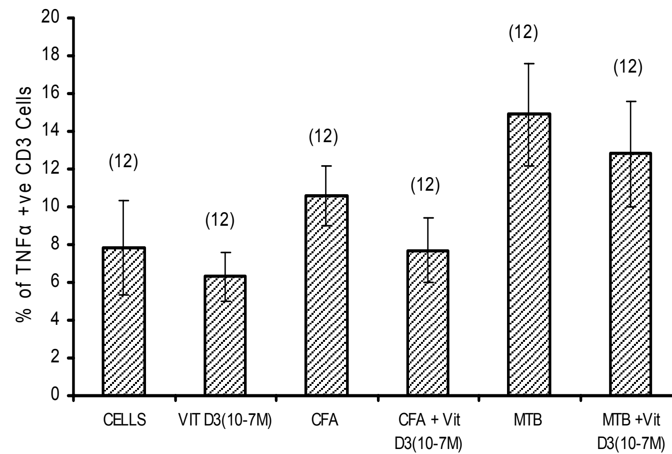
Methods:

Study subjects include 70 pulmonary TB patients and 70 normal healthy subjects. Enumeration of TNF-α and IFNγ positive cells will be done in PBMC cultures by flow cytometry at various time points. Portion of the PBMCs will be used for VDR protein assay and DNA extraction for genotyping of VDR. Serum vitamin D_3 will be estimated using commercial ELISA kits.

Results:

The effect of vitamin D_3 on TNF-α cytokine expression at the intracellular level has been studied in 12 normal healthy subjects so far. A trend towards a suppressive effect of vitamin D_3 was observed on TNF-α expression in CD3 positive cells stimulated with live *M.tuberculosis* and culture filtrate antigen (Fig 6.19). Vitamin D receptor protein assay has been standardized. The study is in progress.

Fig.6.19: Effect of vitamin D_3 on intracellular TNF-α positive CD3 cells in NHS



(PI: Dr.P.Selvaraj; selvarajp@trcchennai.in)

Cytokine gene polymorphisms and cytokine levels in pulmonary tuberculosis

Background:

Th1 and Th2 cytokines play an important role in the immune response against TB and alteration in their levels contribute to the out come of the infection. Single nucleotide polymorphisms in the cytokine genes may influence the cytokine levels in pulmonary TB which may be associated with susceptibility or resistance to TB.

Aim:

- To find out whether variant genotypes of cytokine genes and cytokine levels are associated with susceptibility or resistance to pulmonary TB

Methods:

The study subjects include 150 pulmonary TB patients and 150 normal healthy subjects. Genotyping will be done by PCR- allele specific primer (PCR-ASP), PCR- allele specific oligo nucleotide probe (PCR-ASOP) and PCR-RFLP method.

Results:

Among the various cytokine gene polymorphisms studied, none of the variant genotypes of IL-8 gene (-251 T→A) was found to be associated with susceptibility to TB. TT genotype was associated with higher IL-8 production ($p=0.05$). There was a lack of association of Interferon γ +874A polymorphism with TB. Significantly increased frequency of IL-4 CT genotype of (IL-4 -590 C→ T polymorphism) in patients ($p<0.05$) and CC genotype in NHS ($p<0.01$) was observed. No significant difference among the variant genotypes of IL-12p40 and IL-10 gene polymorphisms were noted (Table 6.8). IL-12p40 and IL-10 gene polymorphisms will be done in more number of samples to confirm the results. Studies on IL-18 gene polymorphisms are in progress.

Table 6.8: Variant genotype frequencies of IL-12p40 and IL-10 gene in normal healthy subjects and pulmonary TB patients

IL-12p40 and IL-10 Polymorphisms	Genotypes	Genotype frequency (%)	
		NHS n=70	PTB n=70
IL-12 p40 3'UTR (C/A)	CC	21.42(15)	14.28(10)
	CA	51.42(36)	57.14(40)
	AA	27.14(19)	28.57(20)
IL-10 (-1082 A/G)	AA	51.42(36)	48.57(34)
	AG	40.00(28)	47.14(33)
	GG	8.57(6)	4.28(3)

n= number of subjects. Numbers in parentheses represent subjects positive for each genotype.

(PI: Dr.P.Selvaraj; selvarajp@trcchennai.in)

Evaluation of cellular immune response to HIV-1 infection with special reference to HIV gag epitope mapping among south Indians

Background:

HIV-specific cytotoxic T lymphocytes (CTLs) play a major role in the control of viral replication. However, a complete understanding of the specific epitope targeted by CTLs is essential to assess this antiviral response.

Aim:

- To identify the immunodominant epitopes recognized in HIV-1 infected south Indian population using gag overlapping peptides

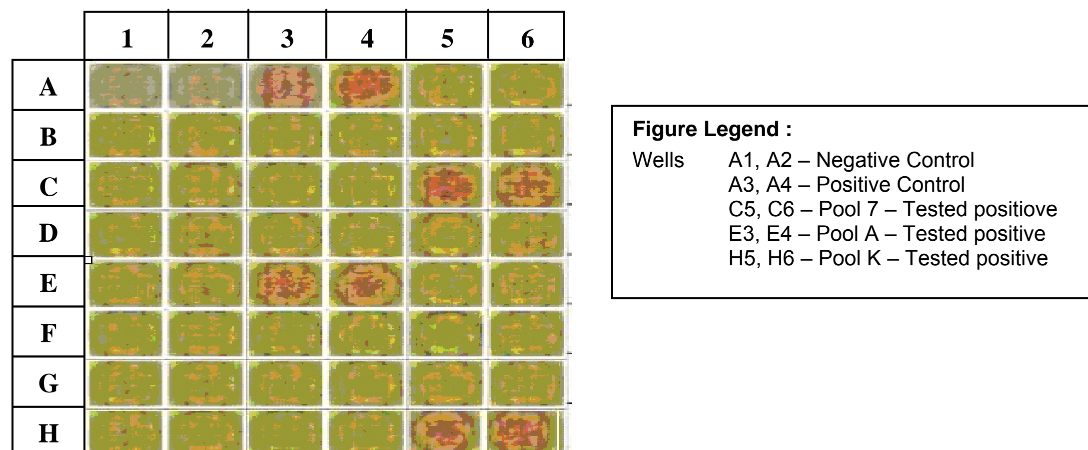
Methods:

A total of 30 HIV infected patients at various stages of diseases have been screened so far. Twenty ml venous blood was obtained from each patient after obtaining written consent. PBMCs were separated by density gradient centrifugation and enzyme linked immunospot (ELISpot) assay was performed using HIV-1 gag peptides obtained from the NIH AIDS repository (overlapping peptides were pooled in such a way that each peptide was found in two peptide pools). The plates were processed further on the next day and subsequently the ELISpots were analyzed on a Carl Zeiss Axioplan ELISpot reader. Peptide matrix technique was used to zero down on the peptide responsible for spot formation. HLA typing of the study subjects will be performed to determine HLA restriction of CTL response.

Results:

We have identified 21-peptide sequences with possible putative epitopes (sequences not shown) (Fig 6.20). The epitopes will be identified after HLA typing and using the Epitope Location Finder (ELF) software.

Fig. 6.20: Representative Elispot assay using HIV-1 gag overlapping peptide pools



Elispot plate showing wells with spots (positive)

PI: Dr.Soumya Swaminathan; soumyas@trcchennai.in)

Effect of anti tuberculosis treatment and antiretroviral therapy on intracellular cytokine production in CD8 T cell subsets of HIV+TB+ patients

Background:

In any infection, naïve CD8 T cells are primed and consequently proliferate and differentiate into effector and memory cells. Peripheral effector CD8 T cells show direct effector function such as cytotoxic activity and cytokine production in response to their targets, whereas memory CD8 T cells do not have direct cytotoxic activity without prior activation but can proliferate and secrete a large amount of cytokines in response to antigenic stimulation. There is a gap of knowledge as to what happens to the CD8 T cell maturation pathway during ATT and ART in HIV+TB+ patients.

Aim:

- To analyze the expression of intra cellular cytokines (IFN-γ IL-2, TNF-α) in phenotypic subsets (naïve, memory, memory effector and effector) of CD8 T cells in patients with HIV and active TB and to study the effect of ATT and ART on cytokine production in different CD8 T cell subpopulations

Methods:

Peripheral blood samples will be obtained from ATT and ART naïve HIV+TB+ patients at baseline (time of entry into the study), 2 months after initiation of ATT and subsequently 1, 4 and 6 months after initiation of ART. Whole blood cells will be stimulated with PMA + ionomycin, purified protein derivative (PPD) and HIV-1 P24 antigen with/without brefeldin A. Cells will subsequently be stained with fluorescence-tagged monoclonal antibodies directed against cell surface markers viz CD8, CD45RA and CCR7 along with internal markers for IFN-γ, IL-2 and TNF-α, Flow cytometric analysis will be performed using a BD FACS calibur. The study is in progress.

(PI: Dr.Soumya Swaminathan; soumyas@trcchennai.in)

Role of chemokines in tuberculous immunity: Role of CXC-type of chemokines and its receptors in tuberculosis

Background:

Pulmonary TB is characterized by granulomatous inflammation with an extensive infiltration of mononuclear cells. The immune response against *M. tuberculosis* is typically T helper 1 (Th1) dominated. It is well established that CD4 T-effector functions. Interferon gamma production, cytolysis of infected monocytes and macrophages etc., are important for bacterial clearance. The recruitment of leukocytes occurs via the regulating chemokines (CXC or CC-type) and its cognate receptors expression on the immune cells is one of the most fundamental host defences for effective containment of the disease. Both CXC – and CC-type of chemokines is recognized as important mediators in a variety of inflammatory states especially during TB. Moreover, the recent studies highlight the importance of CXC type of chemokine in driving the T cells to the site of infection, particularly during TB.

Aim:

- To evaluate and compare expression of selected α-type (IP-10, MIG and IL-8) of chemokines and their receptors (CXCR2, CXCR3) on the immune cells (T cells, B cells, NK cells, monocytes and neutrophils) in the peripheral blood of healthy normal individuals and PTB patients

Methods:

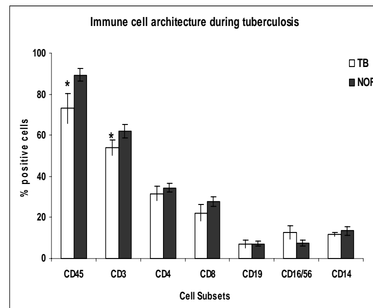
Blood was collected from the normal healthy individuals and PTB patients. The profiling of the immune cells was done by using BD Simultest kit. The cells were dual labeled for various chemokine receptors and analyzed by flow cytometry. Chemokines were assayed in plasma by the cytometric bead array (CBA).

Results:

The cell profile analysis showed a significant decrease only in CD3 T cells (Fig-6.21a) in PTB patients with no significant change in other cell types when compared to normals. The CXCR2 expression was significantly increased on T, NK cells and neutrophils but decreased on monocytes in PTB (Fig.6.21b). The CXCR3 expression was highly significant in PTB patients on CD4 T cells and not

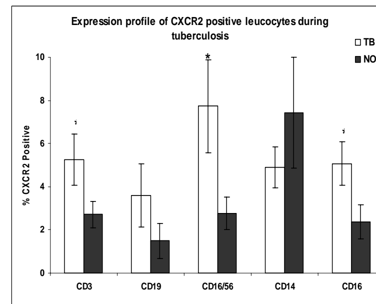
on B cells (fig.6.21c). The IP-10, MIG and IL-8 levels were significantly higher in PTB patients (fig.6.21d).

Fig. 6.21a



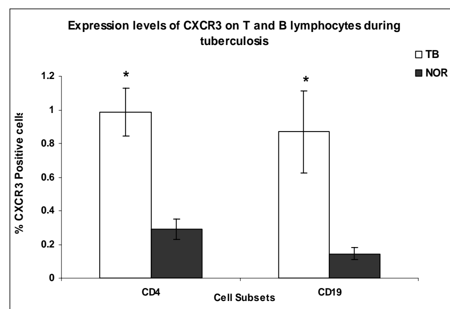
*(p<0.05 denotes the statistical significance between normal and PTB subjects.

Fig. 6.21b



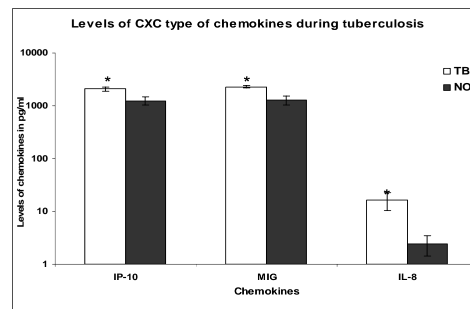
* (p<0.05) denotes the statistical significance between normal and PTB subjects.

Fig. 6.21c



* (p<0.05) denotes the statistical significance between normal and PTB subjects.

Fig. 6.21d:



* (p<0.05) denotes the statistical significance between normal and PTB subjects.

Conclusion:

Our results demonstrate that the CXC type (IP-10, MIG and IL-8) of chemokines and their receptors (CXCR2 and CXCR3) play an essential role in driving the specific immune effector cells to the site of infection to confine the bacilli within the granuloma and combat the disease progression.

(PI: Dr.Sulochana Das; sulochanad@trcchennai.in)

Role of dendtritic cells in mycobacterial immunity: Modulated function of monocyte derived dendritic cells in pulmonary tuberculosis

Background:

Tuberculosis remains one of the challenging diseases to mankind. Despite continuous surveillance and treatment, it is causing a mortality rate of 2 million per year. This failure in prevention of the disease is due to poor understanding of the interaction between host immune and mycobacterial virulence factors. In the immune system, dendritic cells (DC) play a dual role in the immune response, participating in its induction, and the maintenance of immune tolerance. They are likely to play an important and unique role in the generation of protective immunity to mycobacteria. Dendritic cells are derived from different cellular origin of which monocytes derived DCs (MODC) contribute considerably to the circulating DCs. These DCs when encounter an antigen lead to activation and migration to secondary lymphoid organ where they trigger a specific Tcell response.

Aim:

- To perform phenotypic and functional analysis of MODC generated *in vitro* in the presence of GM-CSF and IL-4 from PTB patients and to evaluate their maturation status with lipopolysaccharide (LPS) stimuli.

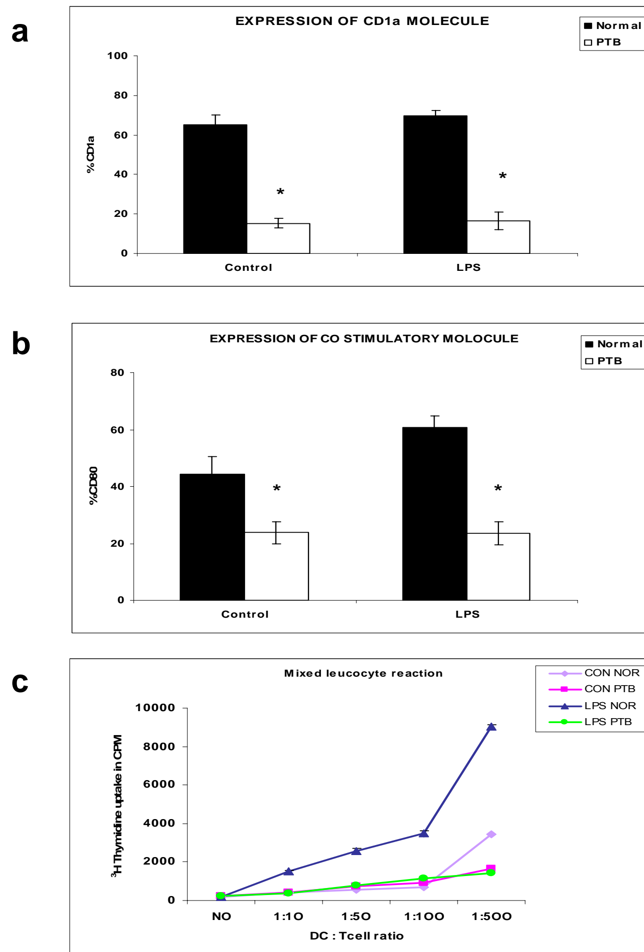
Methods:

Peripheral blood mononuclear cells were isolated by density gradient centrifugation from the blood of recruited healthy individuals and PTB patients. Monocytes were purified using anti-CD14 conjugated magnetic beads. MODC were generated by culturing $CD14^+$ cells with GM-CSF and IL-4 for 6 days. On day 7, the MODC were either stimulated with or without LPS treatment. After 24 hours, DCs were harvested and analyzed using FACS for various phenotypic markers. ELISA for cytokines was performed in the culture supernatants. The infected MODC were subjected to allogenic mixed leucocytes reaction to determine the activation of T cells by MODC.

Results:

The expression of CD1a was markedly lesser in MODC of PTB patients compared to normal individuals but the expression of CD14 was almost absent in the MODC of both subjects (Fig.6.22a). These cells also showed lower levels of HLA class II and CD80 (B7.1) in both control and LPS stimulated group with respect to patient's MODC (Fig.6.22b). Surprisingly the levels of CD86 were up-regulated in differentiated-DCs of PTB patients. Interestingly, MODC derived from PTB subjects revealed an impaired antigen-presentation function as assessed by the reduced capability to induce proliferation of T lymphocytes (Fig.6.22c). These results were then correlated with pro-inflammatory and Th1 cytokines.

Fig 6.22a, b, c



The statistical significance is shown as * ($p<0.05$) compared to normal and PTB.

Conclusion:

Our results support the notion that there is an interference with monocyte differentiation into fully competent DC in PTB patinets. This interference may be due to the exposure of the precursor cells with *M. tuberculosis* antigen. Hence may be an evasion mechanism of *M. tuberculosis* that could contribute to its intracellular persistence avoiding immune recognition.

(PI: Dr.Sulochana Das; sulochanad@trcchennai.in)

Activation of mitogen activated protein kinases by *M. tuberculosis* strains in monocytes/macrophages

Background:

During the early stages of *M. tuberculosis* infection, the control of the intracellular bacterial survival and proliferation is dependent on the macrophage innate resistance. Both mouse and human studies have provided ample evidence that even in the face of an adequate immune response, mycobacteria like *M. tuberculosis* and *M. avium* are able to persist inside macrophages. Of interest, several strains and distinct morphotypes of *M.avium* differ with respect to virulence and persistence in an *in vivo* infection model. Apart from the other vital evasion mechanisms mentioned above, one potential mechanism by which virulent mycobacterial strains, as opposed to avirulent strains, may achieve a state of long-term persistence is the modulation of signaling cascades leading to macrophage activation. Among the diverse signaling cascades that are involved in triggering cellular responses to pathogenic organisms, one essential branch of cell signaling in eukaryotic organisms is the ubiquitously expressed family of mitogen activated protein kinases (MAPK). The MAPKs are serine/threonine protein kinases that respond to a range of external and intracellular stimuli and coordinate signals for diverse cellular functions from cellular growth and proliferation to apoptosis and extracellular-signal regulated kinase (ERK) which has p44 (ERK1) and p42 (ERK2) isoforms. The JNK and p38 are primarily induced in response to cellular stress, osmolarity, heat shock, UV irradiation and also in response to inflammatory cytokines. ERK is mainly activated by growth factors and phorbol esters.

Aim:

- To study the host signal transduction pathways induced in response to *M. tuberculosis* infection

Methods:

The present study mainly focuses on finding out the differential induction of the MAPK in the primary human monocytes and THP-1 cell line (human monocytic cell line), upon challenge with *M. tuberculosis* $H_{37}Rv$, $H_{37}Ra$, drug resistant Beijing strain (DRB) and low virulent clinical strains isolated in south India. Results of this study will shed light into the mechanisms of colonization of mycobacteria in monocytes/macrophages and also the specific signaling pathway which it triggers in order to combat the host immune responses.

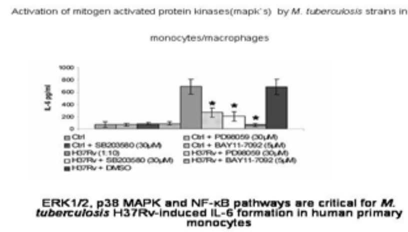
Results:

THP-1 cells were infected with *M. tuberculosis* $H_{37}Rv$, $H_{37}Ra$, DRB and two low virulent clinical strains and the activation of ERK1/2, p38 mapkinases were blotted at 45 min time point.

To decipher the pathway that is utilized by *M. tuberculosis* H37Rv to induce IL-6 cytokine secretion in primary human monocytes, inhibition studies were carried out.

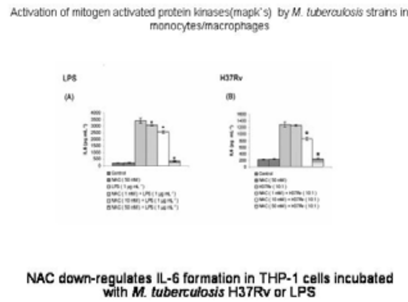
Human primary monocytes were preincubated with pathway inhibitors - 30 µM PD98059 (MEK1 inhibitor), or SB203580 (p38 MAPK inhibitor), or 5 µM Bay 11-7082 (NF-κB inhibitor) for 60 min before incubation with *M. tuberculosis* $H_{37}Rv$ for 24 hr, and cellfree supernatants were assayed for IL-6 secretion. IL-6 production was significantly reduced by all the three inhibitors used — 30 µM PD98059, 30 µM SB203580 and 5 µM Bay 11-7082 inhibited 61.6, 70.39 and 90% respectively. These results show that ERK1/2, p38 MAPK and NF-κB are involved in the signaling of IL-6 production during mycobacterial infection of human primary monocytes (Fig.6.23, 6.24, 6.25)

Fig 6.23 :



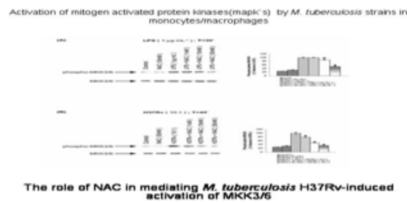
ERK1/2, p38 MAPK and NF-κB pathways are critical for *M. tuberculosis* H37Rv-induced IL-6 formation in human primary monocytes

Fig 6.24 :



NAC down-regulates IL-6 formation in THP-1 cells incubated with *M. tuberculosis* H37Rv or LPS

Fig. 6.25 :



The role of NAC in mediating *M. tuberculosis* H37Rv-induced activation of MKK3/6

Conclusion:

The activation of MAPKs by different strains was different. It has also been shown that ERK1/2, p38 MAPK and NF-κB are involved in the signaling of IL-6 production during mycobacterial infection of human primary monocytes.

(PI : Dr. Sujatha Narayanan; sujathan@trcchennai.in)

Recombinant expression, purification and characterization of low molecular weight penicillin binding protein DacB2 of *Mycobacterium tuberculosis*

Background:

The biosynthesis of bacterial cell wall peptidoglycan involves several cytoplasmic and membrane bound enzymes, which include the Penicillin binding proteins (PBPs). The PBPs are the target enzymes for beta-lactam antibiotics. They are members of the penicilloyl serine transferase family of enzymes, which carry a serine residue at the active site and are characterized by structural motifs unique to each category. PBPs play an essential role in cell division and morphology. The membrane bound PBPs can be broadly classified into the high-molecular weight (HMW) and low molecular weight (LMW) PBPs. The HMW PBPs appear to be important in cell elongation, septation, or shape determinants. The LMW PBPs usually catalyze acyl transfer reactions. The DacB2 protein is the low molecular weight PBP and the function of this is unknown.

Aim:

- To purify and characterize the DacB2 protein of *M. tuberculosis*

Results:

The dacB2 gene of *M. tuberculosis* was PCR amplified (870bp) and it was cloned into PET 43.1 vector. The clones were confirmed by restriction enzyme digestion. One clone (BA6) was chosen for induction studies. The recombinant protein consists of the *nus* and other tags (67kDa) and the Dacb2 protein (33kDa). The expressed protein was confirmed by Western blot. The recombinant protein was purified using Ni-NTA agarose columns specific for the 6XHis tag present in the expressed protein. The pencillinase activity of expressed recombinant DacB2 was carried out by with ^{14}C benzyl penicillin. The assay was performed with different concentrations of protein and ^{14}C benzyl penicillin. The concentration of ^{14}C benzyl penicillin was standardized to 1 µCi per reaction. At this concentration the dialyzed samples gave moderate signals after 10 days exposure.

Conclusion:

The result shows that expressed recombinant DacB2 protein of *M. tuberculosis* is functionally active. Future studies involves site directed mutagenesis in serine residue of DacB2 and protein protein interaction, speculated to regulate the activity of β-lactamases and related proteins like penicillin binding proteins thus indirectly controlling cell viability, cell shape or morphology.

(PI : Dr. Sujatha Narayanan; sujathan@trcchennai.in)

Recombinant BCG vaccine using epitope delivery system for *Mycobacterium tuberculosis*

Background:

One of the highest priorities of TB research is a vaccine more potent in humans than the current vaccine, BCG. Despite widespread use of BCG, TB continues to ravage humanity. Each year, *M. tuberculosis*, the primary causative agent of TB, causes approximately 8 million new cases of TB and 2 million deaths, making it the world's most lethal infectious agent. Adding to the problem, strains of *M. tuberculosis* resistant to the major antibiotics used to treat TB are emerging rapidly worldwide.

Viable carrier systems offer the greatest potential for innovative approaches to develop polyvalent vaccines. Efficient protection against infectious agents often requires the action of both humoral and cellular immune mechanisms. Therefore, an ideal polyvalent antigen delivery system should be capable of stimulating all desired effector cell populations of the immune system. Live replicating bacteria and viruses that stimulate complex immune responses have been rendered avirulent and endowed with the ability to express foreign proteins derived from pathogenic microorganisms

In the previous study we expressed HIV-1 PND epitope in BCG in the carrier antigen background. Two versions of the Cpn10-PND chimeric antigens were constructed and expressed in *M. smegmatis:* 1. the replacement chimera where the PND epitope replaces the Cpn10 loop and 2. the insertion chimera where the PND epitope is inserted into the Cpn10 loop. The expression profiling was analysed in *E. coli*, *M. smegmatis* and BCG which showed mycobacteria specific

expression. The construct was then electroporated into BCG Pasteur. Sub-cellular localization showed that the chimeric antigen was present in the culture supernatant and the cell wall. The immunogenicity of the recombinant BCG (y BCG) was evaluated in a murine model. Vaccination with rBCG expressing the PND epitope induced both cellular and humoral immune responses as measured by lymphocyte proliferation, delayed-type hypersensitivity (DTH) reaction, cytokine secretion, generation of memory T- cells and antibody production.

Aim:

- To express *M. tuberculosis* specific epitopes in BCG using the epitope delivery system
- To evaluate the protective efficacy and immunogenicity of the rBCG constructs in a suitable model of infection.

Results:

Understanding the nature of the host–pathogen interactions at different stages of TB is central to new strategies for developing chemotherapies and vaccines. The antigens were selected based on their role at different stages of *M. tuberculosis* pathogenesis. The antigens included RD1 antigens, mammalian cell entry protein, markers for latency (icl, hsp X) and genes upregulated under *in vivo* conditions in a mouse model of infection. The putative promiscuous and high affinity binding to Class I/II major histocompatibility complex (MHC) molecules were selected based on literature search and by bioinformatics epitope prediction, BIMAS (Bioinformatics And Molecular Analysis Section) (http://thr.cit.nih.gov/molbio/hla_bind/} which ranks potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Applied Biosystems. In this algorithm, binding of the epitope to 33 different human MHC class I molecule and 9 different H2 alleles of mouse is estimated.

The epitopes for inclusion into the epitope delivery system were selected based on the following criteria:

- High affinity binding to MHC allele

- Promiscuity of the epitope
- Binding of the epitope to the frequently occurring MHC allele
- Function of the protein
- If exported or intracellular or transmembrane
- Overall percentage of the protein in whole cell lysate

(PI : Dr. Sujatha Narayanan; : sujathan@trcchennai.in)

Molecular Characterization of *cis* and *trans* acting elements of Acetamidase Operon of *Mycobacterium smegmatis*

Background:

Studying mycobacterial gene regulation at the promoter level is an important goal in the mycobacterial genetics. It is essential to understand the gene expression machineries of mycobacteria related to transcription mechanisms, since many of the mycobacterial genes are not successfully expressed by the well established *E. coli* promoters. Molecular characterization of the many mycobacterial genes and assessment of the drug susceptibility and sensitivity for clinical isolates were hampered by the lack of good homologous mycobacterial expression vectors. The highly inducible enzyme, acetamidase of *M. smegmatis* enables the organism to utilize several amide compounds as sole carbon source including acetamide and formamide. This is expressed in basal level in non-induced conditions and 100 fold induced in the presence of an inducer like acetamide. This enzyme is a part of an operon, the acetamidase operon of *M. smegmatis* which has other four predicted open reading frames (ORFs), which could be involved in the expression and regulation of this operon.

Aim:

- To characterize the promoter of this operon and to construct a stable and suitable expression vector for mycobacteria.

Results:

The predicted four ORFs (Orf C, 1, 2 and 3) upstream to the acetamidase enzyme were cloned and two of which were successfully expressed in *E .coli* expression system and purified. Orf 1 was speculated to be a MarR family of

repressor and it is predicted to have a Helix-turn-helix motif and Orf 2 is predicted to have a zinc finger motif. These two proteins were assessed of their ability to bind with the *cis* acting elements of the operon by Electro Mobility Shift Assays (EMSA) (Fig. 6.26, 6.27, 6.28).

Fig. 6.26: Expression kinetics of pETOrf1 in *E. coli*BL21 (DE3)

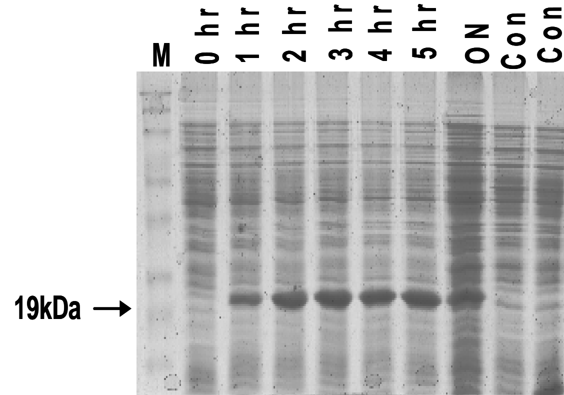


Fig. 6.27: Expression profile of pETOrf2 in *E. coli*BL21 (DE3)

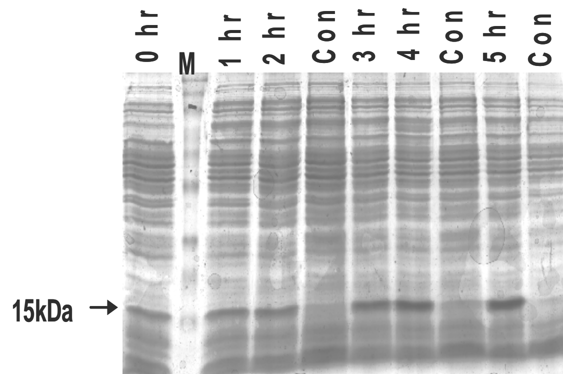
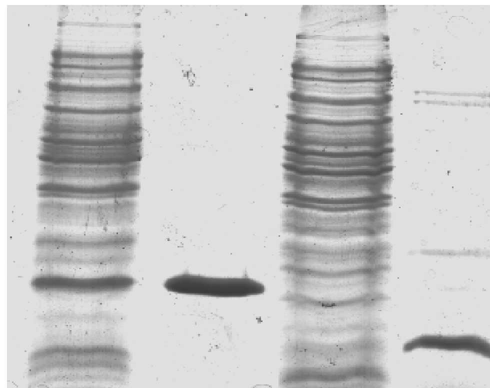


Fig. 6.28: Immobilized Metal Affinity column Chromatographic Purification of AmiA and AmiD



Lane 1 - Crude lysate of pETOrf 1/ BL21 (DE3), lane 2 – AmiA Purified protein, lane 3 - Crude lysate of pETOrf 2/ BL21 (DE3), lane 4 – AmiD Pruified protein

Conclusion:

One of the expressed proteins binds to the various upstream sequence of the acetamidase operon. Foot printing analysis will be carried out to find the exact DNA binding site for these proteins on the operon and other protein science tools like Circular dichroism spectroscopy would be used to characterize these proteins.

(PI : Dr. Sujatha Narayanan; sujathan@trcchennai.in)

In vitro **studies on serine / threonine protein kinase PknE from *Mycobacterium tuberculosis*** $H_{37}Rv$

Background:

Protein kinases play a cardinal role in signal-transduction pathways executing different cellular activities such as adaptation and differentiation in prokaryotes and eukaryotes. Signal transduction in prokaryotes usually use histidine kinases whereas in eukaryotes it is mediated by Ser/Thr or Tyr kinases. Interestingly, analysis of genome sequences revealed the presence of putative genes encoding eukaryotic-type Ser/Thr kinases (STPKs) in many bacterial species and in *M. tuberculosis* genome there are 11 STPKs among which PknE is of our interest. In our previous report we have shown that the gene disrupted mutant has less intracellular survival and shown resistance to various compounds especially to nitric oxide donors.

Aims:

- To study the role of PknE gene in cellular processes – mechanisms of cytotoxicity (apoptosis and necrosis) and proliferation
- To estimate the cytokine secretion, nitrite and glutathione levels after macrophage infection

Methods:

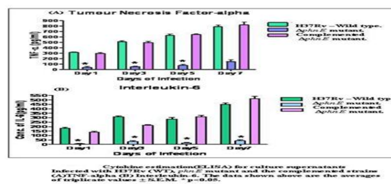
THP-1 cells were differentiated in to macrophages by PMA and subsequently infected with *M.tuberculosis* $H_{37}Rv$ [wildtype], ▲E, C▲E (complemented PknE) for 1st, 3rd, 5th, 7th days. The infected culture supernatants were used for cytokine profiling namely TNF-α, IL-6, IL-4, IFN - γ, IL-10, IL-12p70 by ELISA. The cells were used for AnnexinV – propridium iodide (PI) staining and tunnel assay. To assess necrosis, lactate dehydrogenase assay was done and the nitrite levels were estimated by Griess assay.

Results:

The levels of IL-4, IFN -r, IL-10, IL-12p70 were not significantly different between the wild type and mutant strain of *M.tuberculosis*. The levels of TNF-α, IL-6, levels were significantly reduced in the mutant (Fig-6.29). TNF-α, IL-6, are important pro inflammatory cytokines where TNF is involved in inducing apoptosis and IL-6 in provoking IFN–r response. AnnexinV –PI staining detected early apoptotic events in infected cells. Tunnel assay was used to validate late apoptotic events [Fig-6.30]. Both showed increasing apoptotic events with reference to PknE. LDH assay showed that mutants released low levels of LDH confirming the apoptotic assays done. Proliferation marker staining showed decreased proliferation indicating that apoptosis is occurring. Griess assay showed no difference in the nitrite levels. Glutathione levels were also reduced in the mutant suggesting that the infected cells were dying.

Fig.6.29 :

Fig-1 ***Invitro*** **studies on serine / threonine protein kinase PknE from** ***Mycobacterium tuberculosis*** H37RV



Cytokine estimation(ELISA) for culture supernatants Infected with H37Rv (WT), *pknE* mutant and the complemented strains (A)TNF-alpha (B) Interleukin-6. The data shown above are the averages of triplicate values ± S.E.M. * p<0.05.

Fig.6.30 :

Fig-2 *Invitro* studies on serine / threonine protein kinase PknE from *Mycobacterium tuberculosis* H37RV

TUNEL assay - confirmation of apoptosis by the *pknE* mutant of *M.tuberculosis*. (A)Values were found to be statistically significant $p<0.05$. The results presented are the averages of three independent experiments ± S.E.M. (B) Representative Quadrant analysis of TUNEL positive populations in THP-1 macrophages infected with the three strains on day 5 of infection.Percentages are indicated in the lower right quadrant of every plot.

Conclusion:

The serine threonine kinase Pkn E has a role in apoptosis, nitric oxide metabolism and innate immune response of *M.tuberculosis* infected THP1 macrophage cell line .

(PI: Dr. Sujatha Narayanan; sujathan@trcchennai.in)