

BASIC RESEARCH

Completed studies

Characterization of HIV-1 isolates and pattern of drug resistance from patients initiated antiretroviral therapy

Background

Antiretroviral (ARV) combination therapy is a major advance in the treatment of HIV infection. Two classes of ARVs are now available to treat HIV-1 infection through the NACO-ART program in India (nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors [NNRTIs]), while protease inhibitors have been made available for second line indications. The emergence of resistance to these ARVs is an important cause of treatment failure. The viral mutants exhibit genotypic mutations in reverse transcriptase (RT), protease (prot) and envelope (env) genes. These mutations have been well characterized in subtype B strains, because this subtype is predominant in the western world, where ARVs have been extensively used. In developing countries like India where infections with HIV-1 subtype C is predominant and access to ARVs has been quite recent, it could be possible that the pattern of DRMs are different from that seen in the subtype B isolates. This study will help us to identify mutant genotypes in this population and provide the opportunity for early intervention and effective management.

Aim

- To delineate the DRMs in ART-naïve HIV-positive patients in south India

Methods

Pattern of polymorphism and potential DRMs were evaluated in HIV-1 isolates from 108 HIV-1/TB co-infected individuals naïve to ART, attending the outpatient clinics of the TRC. The samples were subjected to genotyping of HIV-1 RT and prot genes.

Results & Conclusion

One hundred and eight HIV-infected ART-naïve patients with TB were screened for DRMs in the RT and prot genes of the HIV by genotypic method. Of these patients, successful amplification was achieved in 106 patients at baseline (105

for RT and 101 for prot genes). Significant polymorphisms were observed in both RT gene and prot genes in all naïve patients, while one patient each had naturally occurring resistance mutations to NRTI (M184V), NNRTI (Y181C) and protease inhibitor (L90M). Nineteen patients (having viral load >5000 copies/ml plasma) failed therapy at 6 and 12 months of treatment. All patients who failed treatment had resistant mutations to at least one of the RT inhibitors while none of them had mutations in the prot gene. The pattern of DRMs present in the patients failing treatment is presented in the table 16. The commonest mutations observed were M184V (3TC), Y181C (NVP/EFV), and G190A and L74V (NRTIs). The pattern of mutations described on failure of first line ART will be useful in the design of second-line regimens for HIV-infected patients in India.

Table 16: Pattern of drug resistance mutations in patients failing ART

Mutations associated with resistance to NRTI and NNRTI (n=19)																					
CODON	K65	L74				K101		K103	V106		V108		Y181		M184				G190	K219	
AA change	KR	V	L	LV	I	E	KE	N	M	MV	I	IL	C	VY	V	IM	IMV	IV	A	E	N
No. of samples	1	5	1	1	1	2	1	3	4	1	3	1	10	1	11	1	1	1	5	1	1

[Contact person: Dr.Luke Elizabeth Hanna (E-Mail ID: hanna@trcchennai.in)]

Cytokine gene polymorphisms and cytokine levels in pulmonary TB

Background

Th1 and Th2 cytokines play an important role in the immune response against TB and alteration in their levels might contribute to the outcome 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 the influence of variant genotypes of cytokine genes on cytokine levels and their association with pulmonary TB

Methods

The study subjects included 166 pulmonary TB patients (PTB) and 188 normal healthy subjects (NHS). Genotyping of IFN- γ (+874 and +5644), IL-2 (-330 and +160), IL-4 variable number tandem repeat, IL-6 (-174), IL-10 (-1082 and -819) and IL-12B 3'untranslated region +1188 polymorphisms were done using polymerase chain reaction-allele specific primer (PCR-ASP) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. To study the influence of cytokine gene polymorphisms on cytokine levels, phytohaemagglutinin and culture filtrate antigen (CFA) of *M. tuberculosis* induced cytokine levels were measured by ELISA from 72 hour-old peripheral blood mononuclear cell (PBMC) culture supernatants.

Results

Significantly increased frequency of G allele [Odds ratio (OR) 1.46, 95% confidence interval (CI) 1.07-1.98] and decreased frequency of T allele (OR 0.69, 95% CI 0.50-0.94) of IL-2 -330 (T/G) polymorphism was observed among PTB patients compared to NHS ($p=0.017$). The TT genotype of IL-2 -330 polymorphism was significantly under represented in patients as compared to NHS ($p = 0.024$, OR 0.53, 95% CI 0.31-0.92) (table 17). Genotype frequencies of other polymorphisms were not significantly different between NHS and PTB. IL-12p40 levels were significantly lower in NHS with AA genotype than in NHS with AC genotypes ($p<0.05$). In PTB patients, spontaneous IL-12p40 levels were significantly higher among patients with CC genotype compared to patients with other genotypes ($p<0.01$). CFA induced IL-12p40 levels were significantly higher in patients with CC genotype compared to patients with AC genotype ($p<0.05$) (Fig.4). No significant differences in the levels of other cytokines between the variant genotypes of various cytokine gene polymorphisms were observed between NHS and PTB patients.

Conclusions

The present study suggests that the TT genotype of IL-2 -330 polymorphism may be associated with protection to PTB. Further, +1188 polymorphism of IL-12B

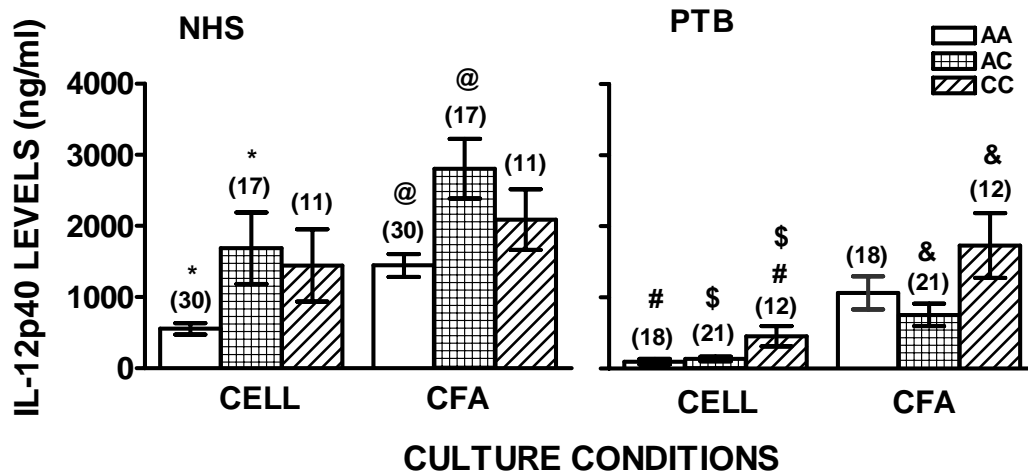
gene either, alone or in combination with closely linked genes may regulate IL-12p40 production and may play a major role on acquired immunity to TB.

Table 17: Allele and genotype frequencies of IL-2 -330 gene polymorphism in NHS and PTB patients

IL-2 gene variants	NHS n = 187	PTB patients N = 164
-330 (T/G) Alleles		
T	0.529	0.436
G	0.471	0.564
Genotypes		
TT	27.8 *	17.1 *
TG	50.3	53.0
GG	21.9	29.9

n = subjects studied; genotype given as percent frequencies.
*p = 0.024; OR 0.53, 95% CI 0.31- 0.92

Fig.4:



Influence of IL-12B gene 3' UTR +1188 polymorphism on spontaneous cell and CFA of *M. tuberculosis* induced IL-12 production in NHS and PTB patients. The values are expressed as mean. The vertical bars represent SE. Numbers in parentheses represent individuals studied. In NHS, AA versus AC * p<0.05, @ p<0.01; In PTB, CC versus AC and AA #p<0.01, \$p<0.01 and CC versus AC &p<0.05.

[Contact person: Dr.P.Selvaraj (E-Mail ID: selvarajp@trchennai.in)]

Human Leucocyte Antigen -DPB1 and vitamin D receptor gene polymorphism studies in HIV and HIV-TB patients

Background

Our earlier studies revealed an association of Human Leucocyte Antigen-A11 (HLA-A11) with resistance and HLA-B40 and -DR2 with susceptibility to HIV and HIV-TB. Other polymorphisms in the HLA and non-HLA regions might also influence susceptibility or resistance to HIV-1 infection and development of TB in HIV-1 infected patients.

Aim

- To find out whether polymorphisms in HLA -DPB1 and vitamin D receptor (VDR) genes are associated with susceptibility or resistance to HIV and HIV-TB

Methods

The study subjects included 131 HIV-positive TB-negative patients (HIV+TB-), 82 HIV-positive pulmonary TB-positive patients (HIV+PTB+), 107 HIV-negative pulmonary TB-positive patients (HIV-PTB+) and 146 healthy controls. HLA -DPB1 typing was done by PCR with locus specific primers followed by hybridization with biotinylated allele specific oligonucleotide probes (ASOP) and detection by chemiluminescence method. VDR gene polymorphisms in the 5' regulatory region (Cdx2 and A-1012G), coding region (*FokI*) and 3' untranslated (UTR) region (*BsmI*, *ApaI* and *TaqI*) were studied using PCR-ASP and PCR-RFLP methods.

Results

HLA-DPB1*1501 was significantly under represented in HIV-PTB+ patients ($p=0.002$, $P_c=0.034$, OR 0.07, 95% CI 0.00-0.49) and HIV+PTB+ patients ($p=0.036$, OR 0.13, 95% CI 0.00-0.92) compared to healthy controls (table 18).

Among the 5' regulatory and coding region polymorphisms of VDR gene, significantly increased frequency of GA genotype of Cdx-2 was observed in HIV+TB- group compared to controls ($p=0.012$, odds ratio (OR) 1.89 95% Confidence interval (CI) 1.14-3.15). In the 3' UTR genotypes, a trend towards

decreased frequency of bb genotype of *BsmI* in HIV+TB- ($p = 0.052$, OR 0.56 95% CI 0.31-1.00) and increased frequencies of AA genotype of *ApaI* in HIV+PTB+ patients ($p=0.056$, OR 1.80 95% CI 0.98-3.30) and tt genotype of *TaqI* in HIV+PTB+ patients ($p=0.05$, OR 2.32 95% CI 0.99-5.46) were observed compared to controls. Haplotype analysis revealed significantly increased frequencies of 3'UTR haplotype B-A-t in HIV+PTB+ group ($P_c = 0.030$, OR 1.75 95% CI 1.14-2.66) and decreased frequencies of b-A-T haplotype in HIV+TB- ($p = 0.031$ OR 0.48 95% CI 0.25-0.89) and HIV+PTB+ groups ($P_c = 0.04$, OR 0.47 95% CI 0.23-0.89) compared to controls (table 19). P_c denotes p value corrected.

Conclusions

The results suggest that HLA -DPB1*1501 may be associated with protection against pulmonary TB development both in HIV-positive and negative subjects. Results on VDR gene polymorphisms suggest that VDR gene 3'UTR haplotype b-A-T may be associated with protection against HIV infection, while B-A-t haplotype might be associated with susceptibility to development of TB in HIV-1 infected patients.

Table 18: Percent frequencies of significant HLA -DPB1 alleles in patient groups & healthy controls

HLA -DPB1 alleles	Healthy controls n=112	HIV+PTB- n=115	HIV+PTB+ n=59	HIV-PTB+ n=110
*0201	23.2 [¶]	28.7	40.7 [¶]	27.3
*1501	11.6 ^{#,§}	8.7	1.7 [§]	0.9 [#]

n= number of individuals studied;

[¶] HIV+PTB+ vs Healthy controls, $p=0.027$, OR 2.27, 95% CI 1.08-4.72.

[#] HIV-PTB+ vs Healthy controls, $p=0.002$, OR 0.07, 95% CI 0.00-0.49.

[§] HIV+PTB+ vs Healthy controls, $p=0.036$, OR 0.13, 95% CI 0.00-0.92.

Table 19: Percent frequencies of selected genotype & haplotypes of VDR gene polymorphisms in HIV-1 infected patients & healthy controls

Genotypes/ haplotypes	Healthy controls n=146	HIV+ TB- n=131	HIV+PTB+ n=82	HIV-PTB+ n=108
<u>Genotypes</u>				
Cdx2 GA	41.1	56.9	48.1	61.2
<i>BsmI</i> bb	30.8	19.9	19.7	26.2
<i>ApaI</i> AA	30.1	26.7	43.8	40.0
<i>TaqI</i> tt	9.6	9.3	19.8	16.5
<u>Haplotypes</u>				
B-A-t	29.0	36.1	41.1	34.6
b-A-T	13.3	6.8	8.0	10.1

n = individuals studied; genotype and haplotype frequencies are given in percentage

[Contact person: Dr.P.Selvaraj (E-Mail ID: selvarajp@trchennai.in)]

Levels of complement in TB

It has been shown that there is hypercatabolism of complement system, increase in circulating immune complex levels along with a reduction in erythrocyte complement receptor-1 (CR-1) and complement-mediated immune complex solubilization. Further, there is an increase in the HH phenotype of CR-1. It was also found that the addition of complement to peripheral blood leucocytes resulted in a reversal of the cytokine imbalance that is normally observed in patients with pulmonary TB.

[Contact person: Dr.V.D.Ramanathan (E-Mail ID: ramanathanvd@trchennai.in)]

Activation of complement system by genetically modified mycobacteria

It was shown that devR mutant of *M.tuberculosis* was able to activate C3 less, although assembly of C4 or factor B was normal in comparison with the wild type *M. tuberculosis* bacilli. Studies with mutants lacking tyrosine phosphatase genes also indicated that these enzymes probably modulate the production of pro inflammatory cytokines.

[Contact person: Dr.V.D.Ramanathan (E-Mail ID: ramanathanvd@trcchennai.in)]

The role of antibody in the interaction between *M. tuberculosis* and macrophages through the complement system

It was found that the addition of antibodies augmented complement-mediated phagocytosis as well as apoptosis. There was a downregulation of complement-receptor expression on both CD4 and CD8 cells when *M.tuberculosis* opsonized with antibody and complement interacted with them. Further, there was a reduction of TNF- α and an increase in IL-6 levels.

[Contact person: Dr.V.D.Ramanathan (E-Mail ID: ramanathanvd@trcchennai.in)]

Molecular characterization of *cis* and *trans* acting elements of acetamidase operon of *M. smegmatis*

Background

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 enzyme is expressed at basal level in non-induced conditions and 100 fold induced in the presence of an inducer-like acetamide, and can be visualized as 47kDa band in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It is part of an operon, the acetamidase operon of *M. smegmatis* which has other four predicted open reading frames (ORFs), which are supposed to be involved in the regulation of this operon. Earlier we reported the cloning of the predicted four ORFs (AmiC, A,

D and AmiS) upstream to the acetamidase enzyme, and expression and purification of two of these using *E. coli* expression system. Here we report the characterization of a regulatory protein, AmiA which was predicted to be a MarR family of repressor with HTH motif.

Objective

- To characterize the promoter of acetamidase operon

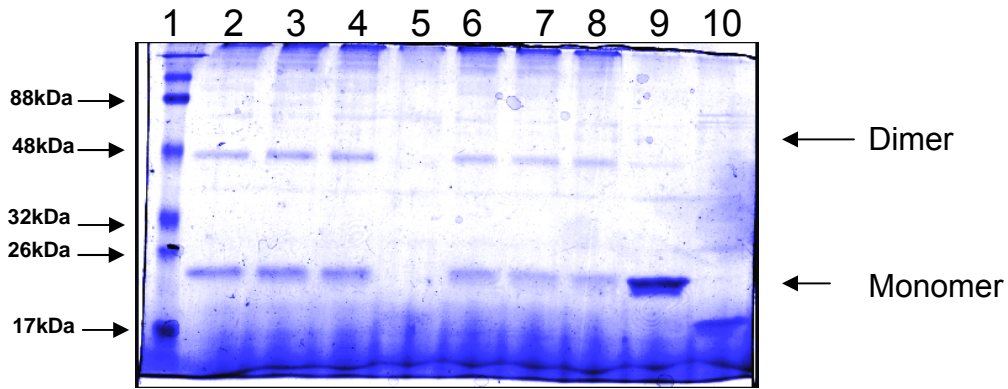
Results

Using glutaraldehyde cross-linking of proteins it was found that the AmiA protein forms a homodimer (Fig.5). Previous studies in our lab signified the possibility of upstream acting elements in the operon. To show the interaction between AmiA, a predicted repressor with these elements, electrophoretic mobility shift assay (EMSA) was performed using the PCR generated and γ -P³²-dCTP labeled DNA fragments. Initially binding ability of this protein within the operon was screened using PCR generated fragments of 300bp length. From this it was established that fragments **L** and **M** which comprises of 1654 – 1975bp and 1776 – 2104bp of the operon, specifically bind with the protein (Fig.6A). The length of this fragment was further minimized to ~ 150 - 200bp and found that the fragment comprising 1776 – 1975bp (Fragment **ML**) of the operon specifically binds with the protein (Fig.6B). Previous studies from our lab showed that this region has promoter activity. It was further confirmed that AmiA acts as a repressor and binds in the operator region of acetamidase operon. Further work would be carried out using footprinting assay to exactly pinpoint the bases involved in binding.

Over expression of AmiC, AmiA and AmiD was attempted using *hsp* promoter driven vector pMV261. Over expression of AmiA in parallel with the wild type operon in *M. smegmatis* greatly influenced the expression of acetamidase. As observed by SDS-PAGE, cells harboring no extra copy of AmiA expressed high level of acetamidase and this increased with increasing concentration of acetamide. However, the cells harboring extra copy of AmiA expressed significantly lower levels of acetamidase than the controls as observed in the protein band intensity at 47kDa (Fig.7). The acetamidase was negatively

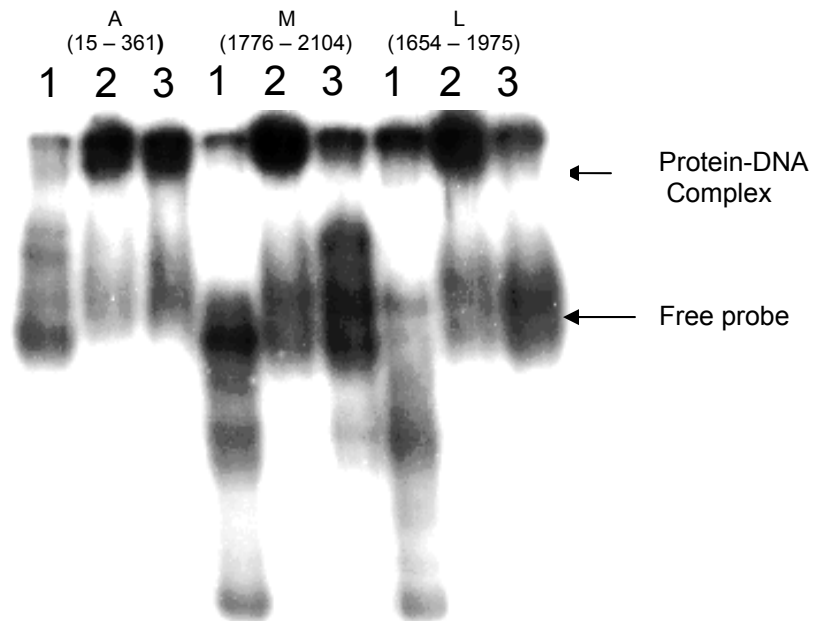
regulated by AmiA, whose over expression abolished the inducibility by acetamide.

Fig.5: Glutaraldehyde cross linking of AmiA



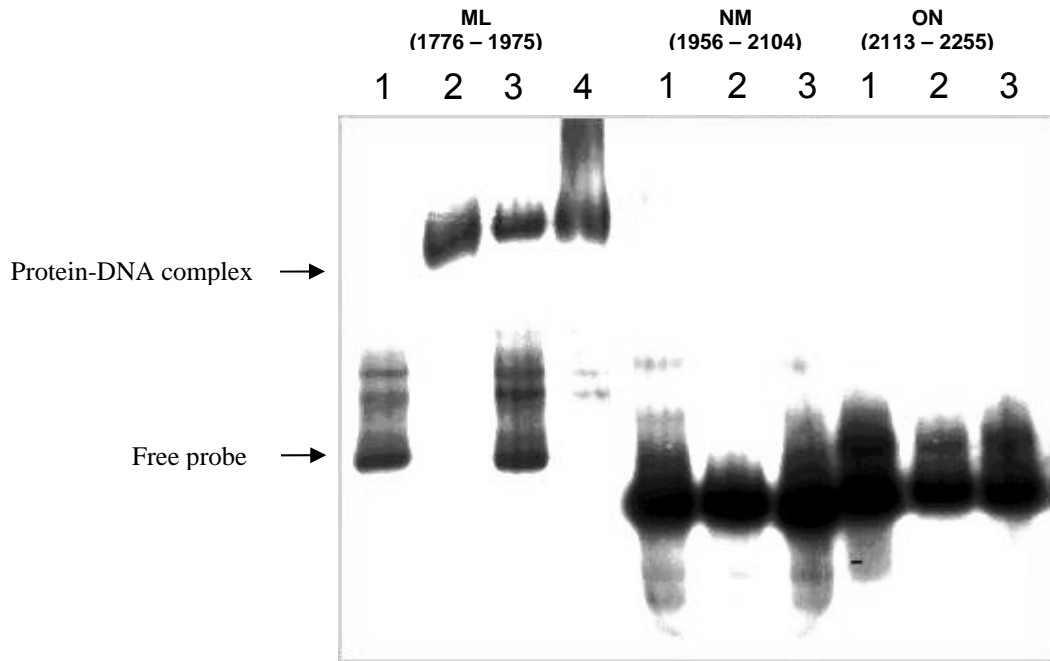
Lane 1: Molecular weight marker, Lanes 2-4: AmiA cross linked with different concentrations of glutaraldehyde, Lane 5 empty, Lanes 6-8 cross linking of AmiA and AmiD, Lanes 9 and 10 has control AmiA and AmiD respectively

Fig.6A: EMSA with ~300bp PCR fragments



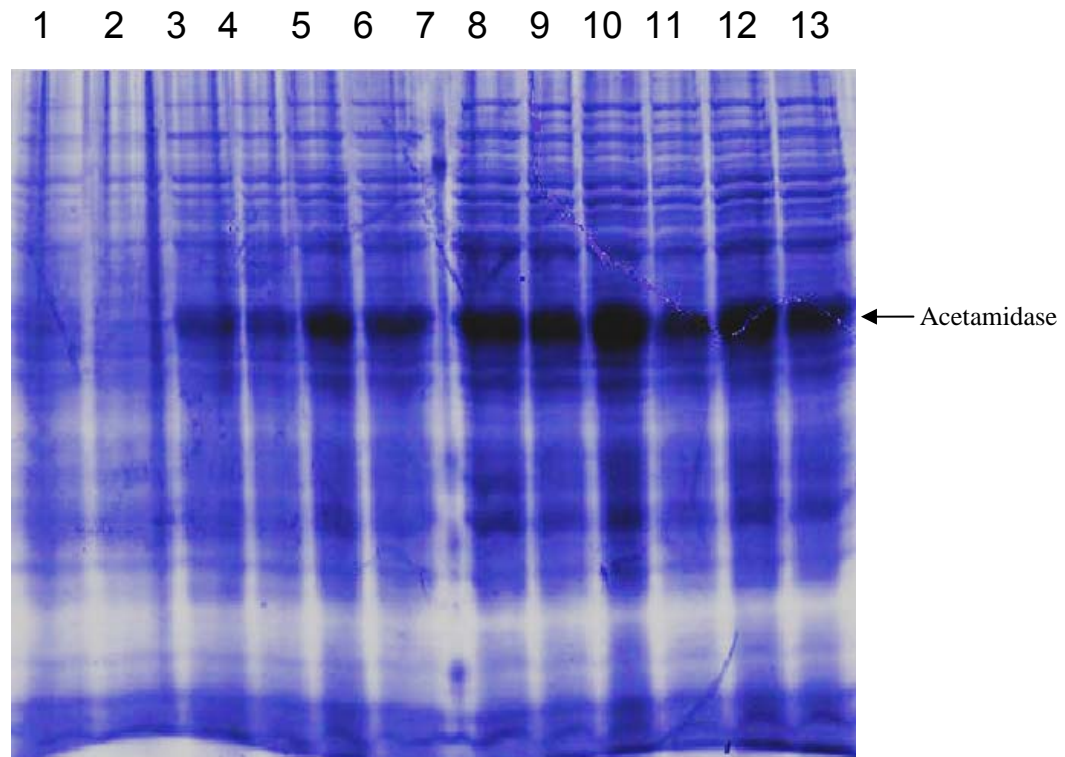
Lane 1: Free probe, Lane 2: 1X, Lane 3: 100X cold chase

Fig.6B: EMSA with ~150 - 200bp PCR fragments



Lane 1: Free probe, Lane 2: 1X, Lane 3: 100X cold chase and Lane 4: nonspecific cold chase

Fig.7: Over expression of AmiA and its effect on the expression of acetamidase



Lane 1: MC² with 0mM acetamide, Lane 2: ORF1 with 0mM acetamide, Lane 3: MC² with 5mM acetamide, Lane 4: ORF1 with 5mM acetamide, Lane 5: MC² with 15mM acetamide, Lane 6: ORF1 with 15mM acetamide, Lane 7: Marker, Lane 8: MC² with 25mM acetamide, Lane 9: ORF1 with 25mM acetamide, Lane 10: MC² with 35mM acetamide, Lane 11: ORF1 with 35mM acetamide, Lane 12: MC² with 50mM acetamide, Lane 13: ORF1 with 50mM acetamide.

Future studies

We are standardizing footprinting analysis to find the exact DNA binding site for AmiA protein on the operon and future experiments are designed to find the protein – protein interaction among the three regulatory proteins of acetamidase operon namely, AmiC, AmiA and AmiD.

[Contact person: Dr.Sujatha Narayanan (E-Mail ID: sujathan@trcchennai.in)]

Mitogen activated protein-erk-kinase activation by different strains of *M. tuberculosis*

Background

Mycobacteria successfully parasitize the host macrophages by inhibiting various host-cell responses. The mycobacteria employ several strategies to subvert host-cell signaling; identification of key molecules involved in signaling, might serve as potential targets for new antimycobacterial therapies. However, the different mechanisms adopted by pathogenic and nonpathogenic mycobacteria in subverting the signaling machinery of host cells are not clear. Any study directed towards delineating the mechanisms that are involved in mycobacterial modulation of macrophage survival and death will provide useful insights in understanding host–mycobacteria interactions. There have been several reports about the role played by signaling pathways in modulating the immune responses. Mitogen activated protein kinases (MAPK) signaling is one of the several pathways which plays a key role in mycobacterial pathogenesis.

Aim

- To study whether the genotype of the *M. tuberculosis* strains influence the cell signaling mechanism

Method

Epidemiologically well characterized strains were chosen and activation kinetics of Mitogen activated protein-erk-kinase (MEK) in THP-1 cells infected with different *M. tuberculosis* strains was studied. The following *M. tuberculosis* strains were used in the study:

- **H₃₇Rv** – virulent laboratory reference strain *M. tuberculosis*
- **H₃₇Ra** – avirulent laboratory reference strain *M. tuberculosis*
- **1338** – clinical isolate obtained during the Model DOTS study conducted at the Bacillus Calmette Guerin (BCG) trial area of Tiruvallur District. This strain has a single copy of the IS6110 insertion sequence and was highly prevalent in the community based on previous RFLP studies. In animal studies, using guinea pig model, this strain had exhibited low virulence.

- **2567 or S7** – clinical isolate obtained during the Model DOTS study conducted at the BCG trial area of Tiruvallur District. This strain has a single copy of the IS6110 insertion sequence and was prevalent in the community, based on previous RFLP studies. It induced the proliferation of predominantly Th2-type cells secreting IL-4, which in turn suppressed Th1 response in healthy subjects.
- **Beijing strain** –Among the isolates screened by spoligotyping from Model DOTS area, a strain with Beijing spoligotype was chosen which was found to be resistant to INH. The prevalence of the Beijing strain in Tiruvallur district is very low (3%).

THP-1 cells were seeded in 24-well tissue culture plates at a density of 0.5×10^6 cells per well and cultured with medium alone (control), or infected with *M. tuberculosis* strains at a multiplicity of infection bacteria: monocyte ratio of 10:1 (or 1 $\mu\text{g/ml}$ Lipopolysaccharide (LPS) derived from *Escherichia coli*, serotype 055:B5 as the positive control) for various periods (15, 30, 45, 60 & 120 min). The amount of phosphorylation of MEK1/2 was determined using western immunoblotting with specific anti-phospho MEK1/2 antibody and densitometry readings.

Results

M. tuberculosis H₃₇Rv induced activation of MEK1/2 at 30 min, caused peak phosphorylation at 45 min (~3.5-fold increase) and the activation signal declined to basal level at 60 min. LPS began its activation at 45 min, peaked at 60 min (~2.5-fold) and reached basal level at 120 min. Activation of Beijing and 1338 strains peaked at 120 min (~2 & 3.5-fold) before which, they had very minimal activation till 60 min. In contrast, strains *M. tuberculosis* H₃₇Ra and 2567 induced phosphorylation as early as 15 min, signal dipped slightly at 45 min and peaked again at 60 min (~3.5 & 3-fold). While the activation signal of *M. tuberculosis* H₃₇Ra had dropped to basal level at 120 min, the phosphorylation signal of 2567 strain still remained high at 120 min (~2.5-fold). Total MEK levels remained consistent throughout the infection, indicating that phosphorylation was specific

to the external stimuli by the mycobacteria (Fig.8 A & B). The activation kinetics of MEK1/2 by all the five strains has been different.

Fig.8: MEK1/2 MAPK activation of THP-1 human monocytes in response to infection with different live *M. tuberculosis* strains

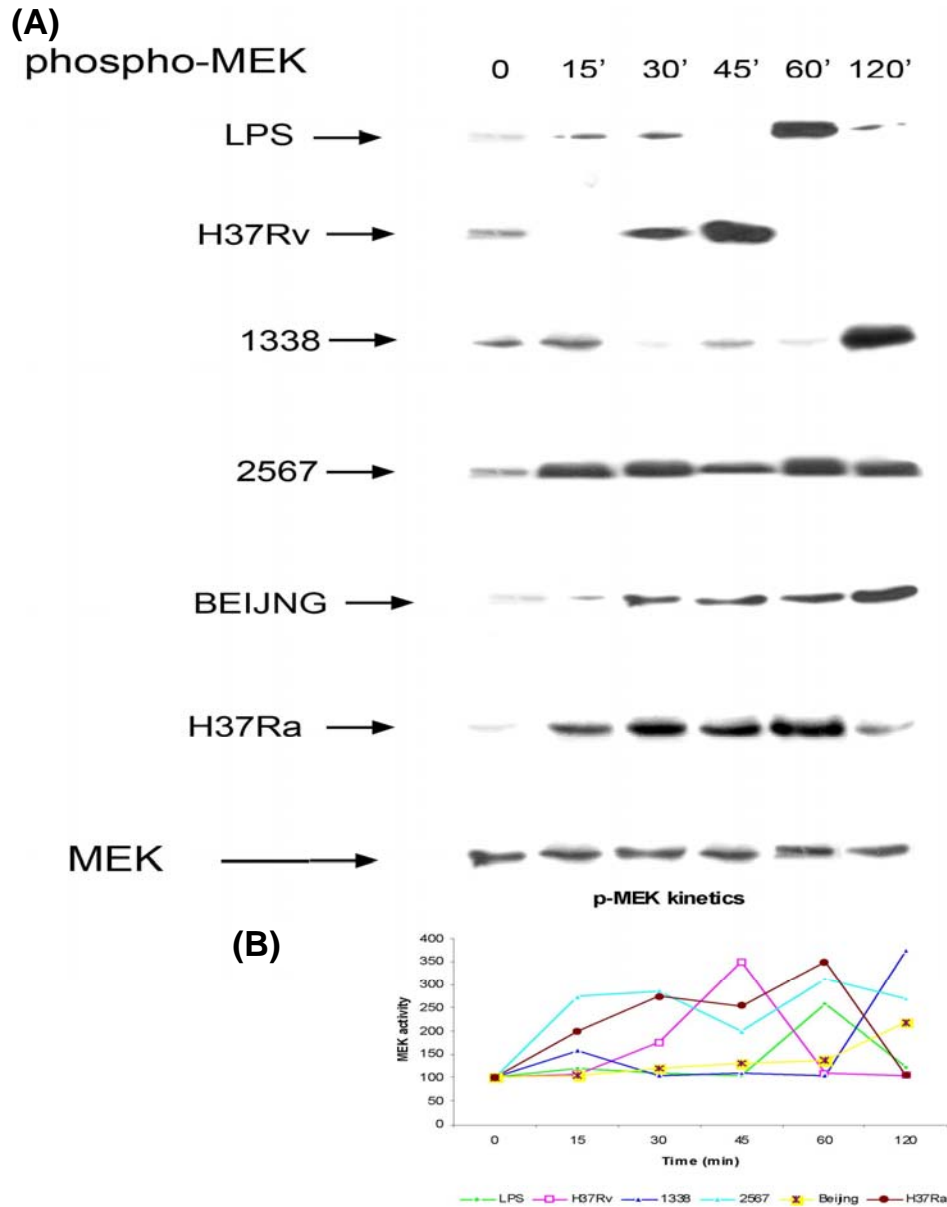


Fig.8 (A) Each of the blots was probed with antibodies against total MEK to ensure equal loading of protein in all the lanes (represented as MEK at bottom).

Fig.8 (B) Corresponding densitometric analyses of blots probed with antiphospho-MEK1/2 antibody. Data shown as mean of three independent experiments performed in triplicate.

Future work

The activation kinetics of p38, JNK, ERK, MKK3/6 in response to infection with the different strains of *M. tuberculosis* will be carried out.

[Contact person: Dr.Sujatha Narayanan (E-Mail ID: sujathan@trcchennai.in)]

***M.tuberculosis* groE promoter controls expression of bicistronic groESL operon and shows differential regulation under stress conditions**

Background

Heat shock promoters of mycobacteria are strong promoters which get rapidly upregulated during macrophage infection and thus serve as valuable candidates for expressing foreign antigens in recombinant BCG vaccine. Heat shock response is characterized by global transcriptional changes including elevated expression of a set of highly conserved genes in response to exposure to a sudden increase in ambient temperature and stress. The mycobacterial Hsp family includes proteins like dnaK, dnaJ, grpE, groES, groEL and other low molecular weight heat shock proteins. Most of these proteins have house-keeping functions and are essential for survival. dnaK, dnaJ, groES, groEL1, groEL2 and acr1 proteins are well-characterized immunodominant antigens of mycobacteria and serve as excellent vaccine candidates

Unlike other bacteria, mycobacteria possess two groEL (groEL1 and groEL2) genes, out of which, the groEL1 is present downstream to that of the groES gene, while groEL2 is present at a different location and is monocistronic. In all mycobacterial species for which genome sequences are available, the organization of groES and groEL1 is identical- upstream groES and downstream groEL1. Thus, it is tempting to speculate that as in other bacteria, groES and groEL1 may form an operon in mycobacteria. But published report has shown by primer extension analysis that groEL1 gene is not co-transcribed with the upstream groES gene, and argued against the operonic organization of groES and groEL1 genes in mycobacteria.

Aims

- Characterization of the *groE* promoter of *M. tuberculosis*
- To determine the promoter region by deletion
- To identify the transcriptional start site (TSS) by primer extension analysis
- To confirm if *groESL1* forms a bicistronic operon by northern blot and RT-PCR
- To explore differential regulation under stress conditions

Methods

The upstream promoter sequences of *groES* gene as well as *groES-EL1* intergenic sequence were PCR amplified and cloned in pJEM13. The promoter activity was evaluated by β -galactosidase assay. The activity of the different upstream promoter constructs was evaluated by β -galactosidase assay under different stress conditions such as temperature shock (42°C and 4°C), pH stress (pH 4 and pH 9), oxidative stress (H₂O₂), SDS stress, osmotic and dehydration stress conditions.

Total ribonucleic acid (RNA) was isolated from *M. tuberculosis* and *M. smegmatis* by Trizol and bead beating method. Primer extension analysis was carried out using MoMuLV RNase H-RT. Heat shock experiment was done by growing *M. tuberculosis* cultures at 37°C till mid-logarithmic phase and then shifting it to 42°C for 1 hr and then shifting back to 30°C for 1hr and reshifting the culture to 42°C for 1hr. Aliquots of culture for isolation of RNA were taken at 37°C, 42°C upshift, 30°C downshift and 42°C upshift.

Northern blot analysis was carried out on *M. tuberculosis* total RNA (50µg) using radiolabelled probe spanning *groES* ORF along with its promoter and downstream sequence by using Rediprime labeling kit, followed by autoradiography.

RT-PCR was done on *M. tuberculosis* total RNA (1µg) by reverse transcription into cDNA using MoMuLV RNase H-RT enzyme at 42°C, for 1hr, using random hexamer primer, followed by PCR amplification using specific primers. For negative control, RT was omitted in the reaction mixture. The RT-PCR product was subjected to electrophoresis on 1.5% agarose gel.

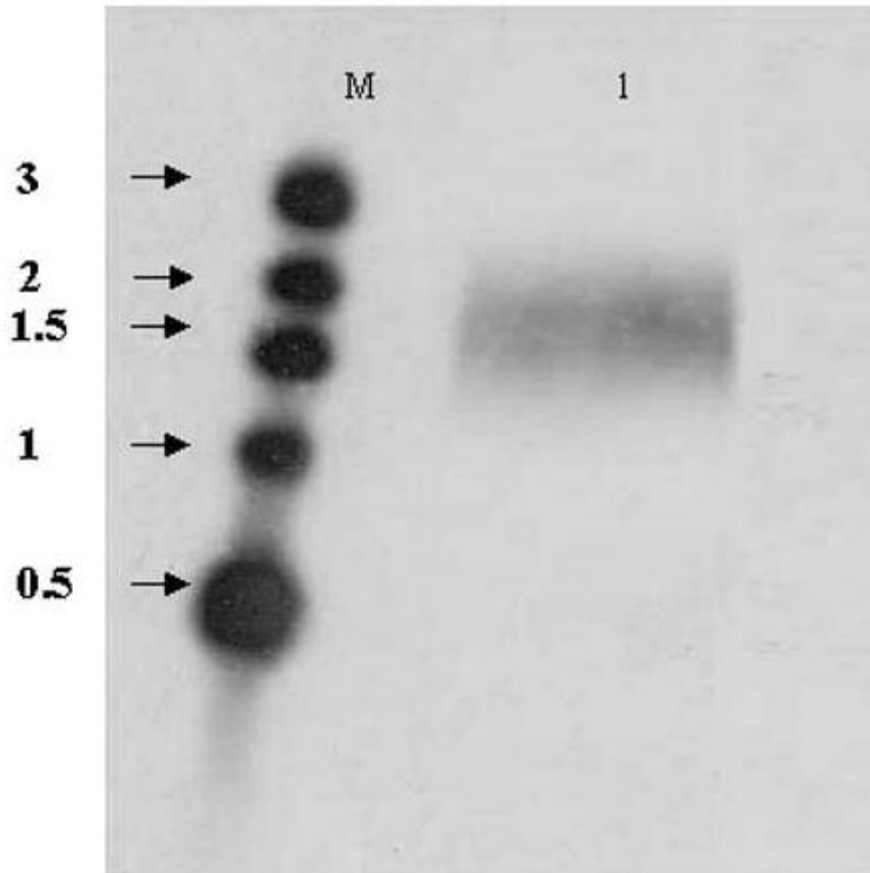
Results

Northern blot analysis was done to confirm the bi-cistronic nature of the *groE* operon. The *groE* promoter, *groES* ORF and the *groES*-*groEL1* intergenic spacer were amplified as a single PCR product and were used as a probe. This probe identified a 2 kb transcript which is consistent with the size predicted for the bi-cistronic *groESL* operon (Fig.9). Further, the same RNA yielded products of sizes 412 bp and 712 bp, by RT-PCR, with primers *va3-gel1R2* and *ups3ap1-gel1R2*, clearly indicating the presence of the *groESL* operon (Fig.10). Primer extension analysis identified two TSS, namely, TSS1 (-236) and TSS2 (-171) out of which one (TSS2) was heat-inducible (Fig.11). Heat shock experiment suggested that the promoter is upregulated at 42°C and gets repressed when shifted to 30°C. Similar effect is not observed for *groEL1* transcripts. The *groE* promoter identified in the study was found to be more active than the *groEL2* promoter in mycobacteria. Further, it was found to be differentially regulated under stress conditions, while the *groEL2* promoter was constitutive.

Future Work

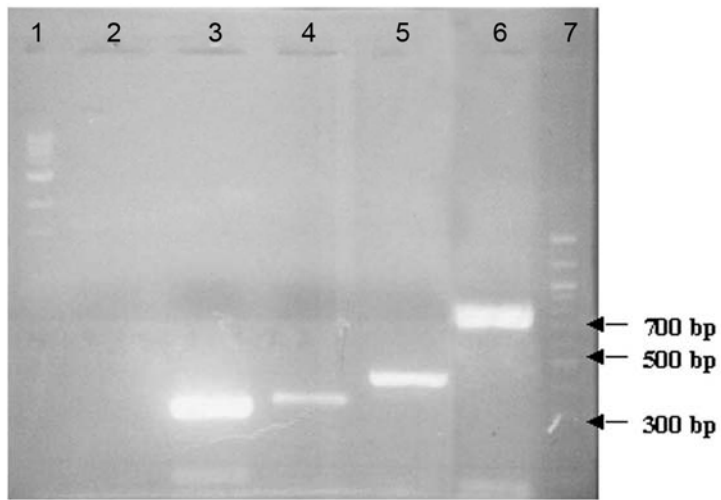
- To study the regulation of the *groE* promoter *in vivo*
- To confirm the controlling inverted repeat of chaperone expression binding elements by EMSA

Fig.9: Northern blot analysis showing the 2 kb bis-cistronic mRNA transcript of groESL operon



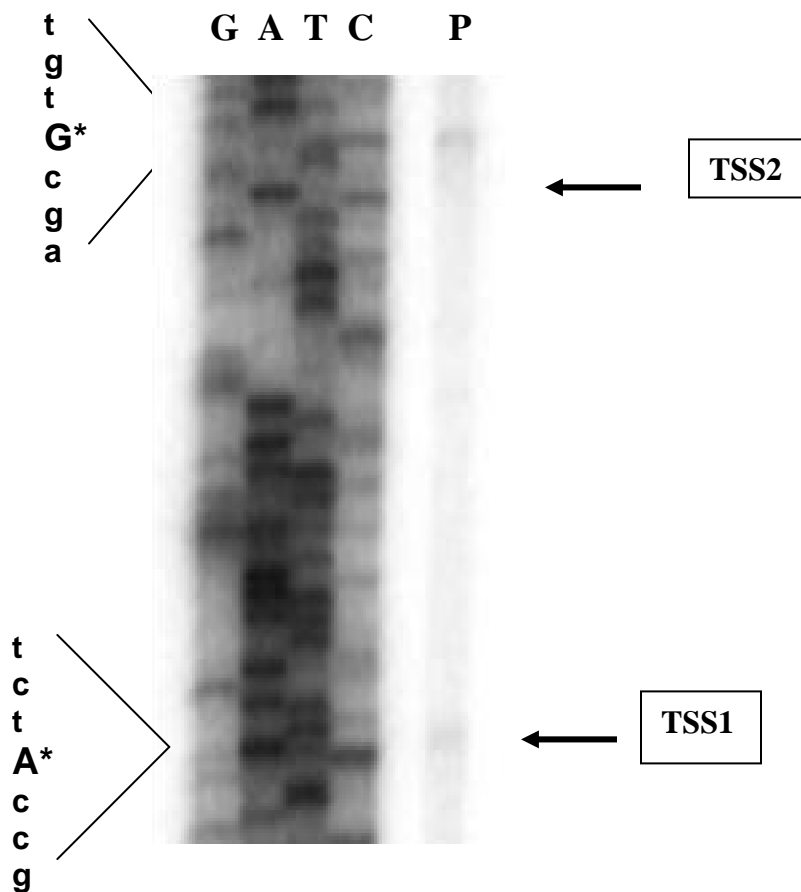
1 μ g of *M. tuberculosis* RNA was fractionated in 1% agrose gel, transferred to a nylon membrane and probed with radiolabelled groES PCR product

Fig.10: RT-PCR analysis on total RNA isolated from *M. tuberculosis*



Lane 1, 1kb Marker (NEB); Lane 2, -RT; Lane3, ups3ap1-upsrkp1 (300 bp); Lane 4, va3- va5 (312bp); Lane 5, va3- gel1R2 (412 bp); Lane 6, ups3ap1-gel1R2 (712 bp) and Lane 7, 100 bp ladder

Fig.11: Identification of groESL TSS by primer extension analysis



Primer extension and DNA sequencing products (Lanes, A, C, G & T) were generated by end-labelled primer. The products were separated on 6% urea-polyacrylamide sequencing gel.

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Global transcriptome profile of PknE deletion mutant from *M. tuberculosis* during THP-1 human macrophage infection

Background

Pathogenicity of mycobacteria is linked to survival within the macrophages in a bactericidal environment. Modulation of host cellular trafficking pathways may be influenced by signal transduction molecules expressed by pathogenic bacteria. The advancement in genomics revealed 11 eukaryotic-like serine/threonine

protein kinases [STPK] in *M. tuberculosis* genome besides two-component system, which perceives the signals to external milieu. We have undertaken studies to characterize the STPK PknE. In the previous report we have showed that the gene-disrupted mutant induced profound apoptosis, and secretion of pro-inflammatory cytokines was impaired.

Aim

- To study the global transcriptome response between the gene-disrupted and wild type (H₃₇Rv) strains infected human macrophage-like THP-1 cells

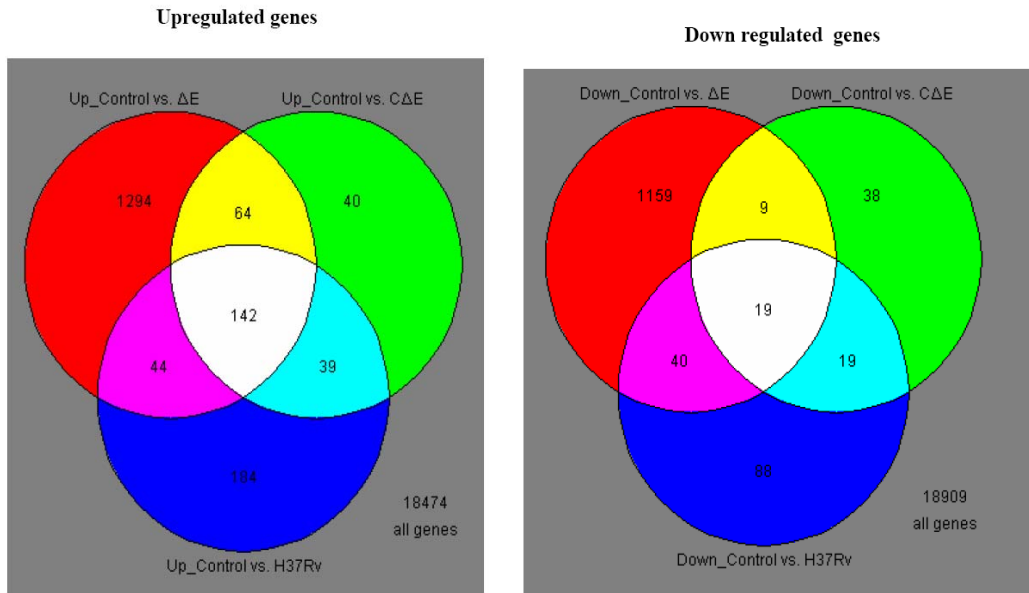
Methods

THP-1, a human monocytic cell line was differentiated into adherent macrophages by the addition of phorbol myrstate acetate (PMA). The macrophages were infected with the strains H₃₇Rv, Δ PknE and complemented Δ PknE. RNA was isolated from uninfected and infected cells after 5 days post infection. The quality of RNA was assessed by NanoDrop® ND-1000 spectrophotometer and Agilent 2100 bioanalyzer. The 22k human microarray was performed in an agilent platform. The data was analyzed using gene spring software.

Results

The experiment revealed several genes that were modulated upon the peak time point of apoptosis (Fig.12). The mutant infected macrophage showed differential expression in the gene families of our interest viz signal transduction (MAPK, JAK-STAT), apoptosis family, immune response genes (cytokines, chemokines) and metabolism. In a holistic view, the results showed that this gene was involved in altering the initial bacilli–host interaction.

Fig.12: Venn diagrams summarizing gene expression observed in the experiment



Summary Venn diagrams:

142 Genes upregulated in all experiments

1294 Genes upregulated in experiments Control vs. ΔE

44 Genes upregulated in experiment Control vs. ΔE and Control vs. H37Rv

184 Genes upregulated in experiment Control vs. H37Rv

39 Genes upregulated in experiment Control vs. H37Rv and Control vs. CΔE

40 Genes upregulated in experiment Control vs. CΔE

64 Genes upregulated in experiments Control vs. ΔE and Control vs. CΔE

Summary Venn diagrams:

19 Genes downregulated in all experiments

1159 Genes downregulated in experiments Control vs. ΔE

40 Genes downregulated in experiment Control vs. ΔE and Control vs. H37Rv

88 Genes downregulated in experiment Control vs. H37Rv

19 Genes downregulated in experiment Control vs. H37Rv and Control vs. CΔE

38 Genes downregulated in experiment Control vs. CΔE

9 Genes downregulated in experiments Control vs. ΔE and Control vs. CΔE

Conclusion

The mutant showed modulatory effects on the host genes involved in apoptosis, immune response, metabolism and signal transduction. Hence it can be predicted that upon infection, PknE is involved in bacilli survival.

[Contact person: Dr.Sujatha Narayanan (E-Mail ID: sujathan@trcchennai.in)]

Characterization of FtsY, a signal particle receptor of *M. tuberculosis*

Background

The ability of *M. tuberculosis* to survive, establish and damage the host tissue depends on multiple factors like cell wall components, secreted proteins and enzymes. The signal recognition particle (SRP) pathway is a universally conserved pathway for co-translational secretion of proteins across membrane. It consists of a cytoplasmic SRP and its membrane bound cognate receptor FtsY. It has already been well established that signal recognition pathway is involved in secretion in streptomyces and bacillus. There is also recent evidence to show that this pathway is required for virulence in *Streptococcus pyogenes*. The role of SRP pathway has not yet been explored in *M. tuberculosis*.

Aim

- To characterize the role of the SRP receptor FtsY of *M. tuberculosis*

Method:

Control (*M.smegmatis* mc² 155 carrying multicopy plasmid pMAC206) and MsRvF (*M.smegmatis* mc² 155 overexpressing *M. tuberculosis* FtsY under inducible acetamide promoter of pMAC206) was grown in LB medium in the presence of kanamycin and induced with 0.2% acetamide for 36 hrs. The cells were pelleted, sonicated and cleared by centrifugation. Cleared lysate was ultracentrifuged at 40000rpm for 1 hr. The cytosolic and membrane fractions were run on 10% SDS PAGE and confirmed by western blot using anti C-terminal His antibody.

For colony morphology studies, 10µl of saturated culture of the strains were spotted on 7H9 plates supplemented with 0.2% acetamide and incubated at 37°C for 4-5 days.

Results

Upon resolving the membrane and cytosolic fraction of crude lysate obtained from MsRvF in 10% SDS-PAGE gel, it was found that majority of the proteins were localized in the membrane fraction (Figs.13 & 14).

Colony morphology studies showed that the control strains grew as smooth, shining, flat, well spread colonies with irregular edges whereas the colonies of MsRvF strains were small, more compact, raised, rough and had a highly wrinkled surface (Fig.15).

Discussion

Like FtsY of other prokaryotes FtsY of *M. tuberculosis* is found to be localized in membrane region of *M.smegmatis* mc²155 upon overexpression. In order to study the phenotypic characteristics of the FtsY gene, it was heterologously overexpressed in *M.smegmatis*. Though it did not affect the overall growth (data not shown), there was considerable difference in colony morphology between the two strains when grown on solid media. The rough and wrinkled surface of MsRvF indicates that over expression of FtsY leads to changes in the cell wall properties most importantly the glycopeptidolipids on the cell surface. These lipids play a major role in the entry of pathogen into host cells and also in biofilm formation.

Fig.13: Localization of the acetamide induced *M. tuberculoiss* FtsY protein in *M.smegmatis* by differential centrifugation partitioning CF, Cytosolic fraction: MF, Membrane fraction

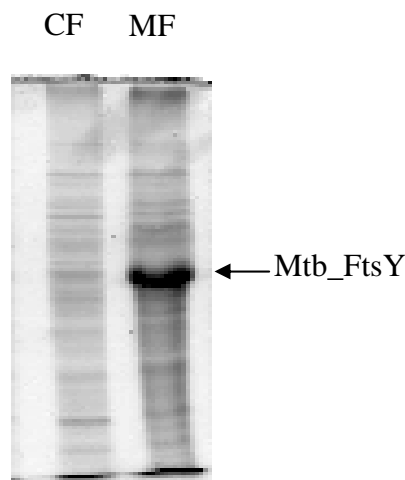


Fig.14: Western blot analysis of localization of the acetamide induced *M. tuberculosis* FtsY protein in *M.smegmatis* by differential centrifugation partitioning. CF cytosolic fraction MF membrane fraction

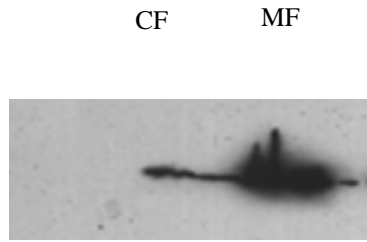
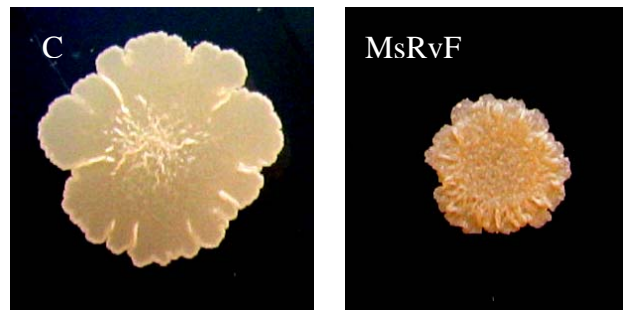


Fig.15: Colony morphology of Control (C) and MsRvF (M) 10 μ l of saturated culture of control and MsRvF were spotted on 0.8% 7H9 agar supplemented with 0.2% acetamide were grown at 37°C for 4-5 days



[Contact person: Dr.Sujatha Narayanan (E-Mail ID: sujathan@trcchennai.in)]

Ongoing studies

Role of variant genotypes of VDR gene on plasma vitamin D₃, VDR expression and intracellular cytokine positive cells in pulmonary TB

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 on plasma vitamin D₃ level, VDR expression and intracellular cytokine positive cells will explore the basic molecular events associated with vitamin D₃ and immunity to TB.

Aim

- To study the regulatory role of variant genotypes of VDR gene on plasma vitamin D₃, VDR expression and intracellular cytokine positive cells in pulmonary TB

Methods

The study population comprised of 70 PTB and 70 NHS. Enumeration of T-cell subsets positive for TNF- α and IFN- γ is being done in PBMC cultures by flow cytometry at various time points. Portion of the PBMCs is used for VDR protein assay and DNA extraction for genotyping of VDR. Plasma vitamin D₃ is estimated using commercial ELISA kits.

Results

The effect of vitamin D₃ on IFN- γ and TNF- α cytokine expression at the intracellular level, VDR expression and vitamin D₃ levels has been studied in 15 NHS and 15 PTB patients so far. A significant suppressive effect of vitamin D₃ was observed on IFN- γ and TNF- α expression in CD4 and CD8 positive cells stimulated with live *M.tuberculosis* and CFA ($p<0.05$). A significantly increased level of 1,25 dihydroxy vitamin D₃ was observed in PTB patients compared to NHS ($p=0.007$) and a decreased VDR protein level (femtomoles/mg total protein) was observed in PTB patients as compared to NHS ($p=0.03$). More number of samples will be studied.

The study is in progress.

[Contact person: Dr.P.Selvaraj (E-Mail ID: selvarajp@trchennai.in)]

Molecular subtyping of HLA -A11, -B40 and -DR2 antigens in HIV and HIV-TB patients of south India

Background

Our earlier studies revealed the association of HLA-A11 with resistance, while HLA-B40 and -DR2 with susceptibility to HIV and HIV-TB. Variability among HLA subtypes are known to influence HIV/AIDS differentially. Hence identification of specific subtype of HLA-A11, -B40 and -DR2 antigens are sought to dissect the role of HLA in influencing HIV and HIV-TB in south Indians.

Aim

- To identify the allelic subtypes of HLA-A11, -B40 and -DR2 antigens that may be associated with susceptibility or resistance to HIV and HIV-TB in a south Indian population

Methods

Molecular subtyping for HLA-A11, -B40 and -DR2 positive subjects among HIV+TB-, HIV+PTB+, HIV-PTB+ and healthy controls is carried out employing PCR based sequence specific oligonucleotide probe method and detection by chemiluminescence.

Results

Preliminary results suggest that HLA-A*1101 is the predominant subtype among HLA-A11.

Further experiments are in progress.

[Contact person: Dr.P.Selvaraj (E-Mail ID: selvarajp@trchennai.in)]

CD209 gene polymorphisms in HIV and HIV-TB patients of south India

Background

Our earlier study has shown that variant genotypes and diplotypes of mannose binding lectin gene are associated with susceptibility to development of TB in HIV-1 infected patients. Polymorphisms in CD209 gene are known to influence

immune responses and might be associated with susceptibility to HIV-1 infection and development of TB in HIV-1 infected patients.

Aim

- To find out whether polymorphisms in CD209 genes are associated with susceptibility or resistance to HIV and HIV-TB

Methods

The study subjects include 131 HIV-positive TB-negative patients (HIV+TB-), 82 HIV-positive patients with pulmonary TB (HIV+PTB+), 107 HIV-negative PTB positive patients (HIV-PTB+) and 146 healthy controls. CD209 exon 4 repeat polymorphism was studied by using PCR method.

Results

The allele 7.5 was found to be the most frequent repeat allele of CD209 exon 4 repeat polymorphism observed in the study subjects with a frequency of more than 99%.

The study is in progress.

[Contact person: Dr.P.Selvaraj (E-Mail ID: selvarajp@trchennai.in)]

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

Background

Immunity to TB is mediated through T-cells, and information on the molecules recognized by the cellular subsets mediating protection is still scarce. The culture filtrate (CF) from *M. tuberculosis* was fractionated by preparative two-dimensional electrophoresis to identify the antigens that strongly stimulate T-cells.

In the previous years' Annual Report (2005-2006 and 2006-2007), a systematic approach was adopted to test the antigens purified by two-dimensional preparative separations, in human subjects and the results of *in vitro* assays have been described. Significantly higher ($p < 0.05$) IFN- γ secretion was induced by 105 fractions in healthy household contacts (HHC) compared to TB patients.

All these fractions were subjected to proteomic analysis using tandem mass spectrometry (ESI LC MS/MS), and characterization of the fractions are presented in this report

Aims

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

Methods

The study subjects are as follows:

- Apparently HHC of sputum positive pulmonary TB living in the same household (TB was ruled out in this group during the time of blood collection and hence considered “protected”)
- Newly diagnosed adult pulmonary TB cases and they form the “susceptible” group

The methods followed are as follows:

Five microgram of protein from each fraction was subjected to overnight digestion with 1µg of trypsin. All the samples were analyzed using Thermo Finnigan LTQ mass spectrometer at Dr. John T. Belisle’s laboratory at Colorado State University, USA.

Results

The data from the protein digestion analyzed by LC MS/MS were searched against the *M. tuberculosis* proteome database using SEQUEST software. All the peptides were assigned to the gene products of *M. tuberculosis*, based on the experimentally derived MS/MS fragmentation pattern. Protein identification by SEQUEST was further validated by Scaffold software. In the 105 fractions which induced significant IFN- γ response in contacts, 57 different proteins were identified. Among these proteins, 35 proteins observed as immunodominant antigens were reported in earlier studies and 22 proteins were novel T-cell antigens identified in our study (table 20).

Generally, in literature low molecular weight proteins (<35kDa) have been described as immunologically active. Our study has identified 12 potential T-cell protein antigens which have a molecular weight >40kDa (FabG4, GgtB, GlnA1 MmsA, Pks13, ProA, Rv0462, Rv2251, Rv2721c, Rv3169, SahH, and Tal).

Some of the novel antigens identified in this study, are being over-expressed as recombinant proteins in *E. coli* and immunologically characterized, so that their potential in subunit vaccine design and specific diagnosis of TB can be evaluated.

Table 20: Novel T-cell antigens identified in IFN- γ inducing 2-D liquid phase electrophoresis fractions of the culture filtrate protein

Protein name	Rv no	Biological function
Can	Rv1475c	TCA cycle aconitase enzyme
AcpM	Rv2244	Involved in fatty acid bioynthesis
Adk	Rv0733	ATP AMP transphosphorlase(adenylate kinase)
Ald	Rv2780	Secreted L-alanine dehydrogenase
FabG4	Rv0242c	Probable 3-oxoacyl-(acyl-carrier protein) reductase
Fba	Rv0363c	Involved in glycolysis (fructose bispophate aldolase)
Frr	Rv2882c	Ribosomal recyclic factor
GgtB	Rv2394	Probable gamma glutamyl transpeptidase precursor
MmsA	Rv0753c	Probable methylmalonate-semialdehyde dehydrogenase
Pgi	Rv0946c	Probable glucose 6 phosphate isomerase
Pks13	Rv3800c	Polyketide synthase
ProA	Rv2427c	Probable gamma glutamyl phosphate reductase protein
Rv1324c	Rv1324c	Possible thioredoxin
Rv1558	Rv1558	Function unknown
Rv1910c	Rv1910c	Function unknown
Rv2204c	Rv2204c	Function unknown
Rv2721c	Rv2721c	Function unknown
Rv3169	Rv3169	Function unknown
Rv3716c	Rv3716c	Function unknown
SahH	Rv3248c	S-adenosyl L-homocysteine hydrolase
Tal	Rv1448c	Probable trans aldolase
TB49.2 or CIP50	Rv0462	Probable protein involved in the phagosomal maturation arrest

[Contact person: Dr. Alamelu Raja (E-mail ID: alamelur@trchennai.in)]

Cytotoxic cell response in *M. tuberculosis* infection

Background

Early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are antigens encoded by ESX-1 locus of *M. tuberculosis*, but absent in *M. bovis* BCG and are useful in diagnosis of tuberculous infection, as well as when incorporated in vaccines. ESAT-6 and CFP-10 are potent T-cell antigens. Studying the role played by specific T-cell subsets in response to overlapping peptides may reveal epitopes in these proteins which might be useful in the diagnosis of TB and in vaccine design.

Aim

- To investigate the IFN- γ and IL-4 responses of CD4 as well as CD8 T-cell responses to overlapping peptides (20-mers overlapping by 10 amino acids) of both ESAT-6 and CFP-10

Methods

The two study groups of this study were healthy household contacts (HHC) (n=20) and pulmonary TB patients (PTB) (n=20).

The IFN- γ as well as IL-4 responses to all the overlapping ESAT-6 peptides and CFP-10 peptides were studied. Diluted whole blood (1:2) of subjects was cultured with the respective ESAT-6, CFP-10 peptides and whole antigens. The intracellular cytokine response was investigated by flow cytometry.

Results

In the previous Annual Report (2006-2007), lymphocyte proliferation to ESAT-6, CFP-10 peptides was shown. We observed peptides Esp1, Esp6, Esp7 and Esp8 among ESAT-6 and the peptides Cfp6, Cfp7, Cfp8 and Cfp9 among CFP-10, to induce more CD4 and CD8 cell proliferation in HHC than PTB. In this report, cytokine production to ESAT-6 and CFP-10 overlapping peptides has been investigated.

The percentage of IFN- γ and IL-4 positive CD4 and CD8 cell induced by the overlapping peptides of both the proteins was measured. When IFN- γ responses in CD4 cells to ESAT-6 peptides was considered, it was found that only the peptides Esp1₁₋₂₀ (p<0.001) and Esp6₅₁₋₇₀ (p<0.01) induced a significantly higher percentage of CD4 positive cells in

HHC when compared to PTB. For the whole ESAT-6 protein the response was significantly elevated in HHC compared to PTB ($p < 0.001$) (Fig.16).

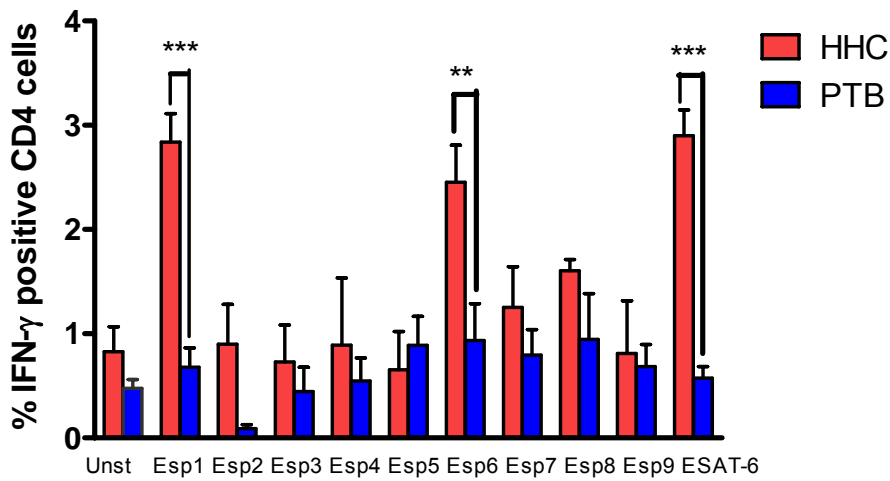
The IFN- γ responses were also studied in CD8 cells to ESAT-6 peptides. It was observed that as in the case of CD4 cells, the peptides Esp1 ($p < 0.001$) and Esp6 ($p < 0.01$) induced significantly higher percentage of IFN- γ positive CD8 T-cells in HHC compared to PTB. No significantly enhanced response was seen for whole CFP-10 protein between HHC and PTB (Fig.17).

Since peptides inducing Th1 cytokines to a greater extent and Th2 cytokines to a lesser extent are considered to be protective, the ratio of IFN- γ to IL-4 was studied for all the ESAT-6 peptides. Of all the peptides, the peptide Esp6 induced an enhanced response ($p < 0.05$) in HHC when compared to PTB. For rest of the peptides and whole ESAT-6 protein, no significant difference in response was observed (Fig.18).

The IFN- γ responses to CFP-10 peptides were studied in CD4 as well as CD8 cells. None of the peptides exhibited a significant response for both the cell types (data not shown). IFN- γ /IL-4 ratio was studied in CD4 and CD8 cells for all the CFP-10 peptides. Although an increased response was observed for the peptides Cfp6₅₁₋₇₀, Cfp7₆₁₋₈₀ and Cfp8₇₁₋₉₀ in HHC and not in PTB, this was not significant (Fig.19).

The ESAT-6 peptide, Esp6₅₁₋₇₀ evinced an enhanced IFN- γ /IL-4 ratio in HHC indicating that it contains a protective epitope which may have implications in vaccine design. None of the CFP-10 peptides displayed a protective response.

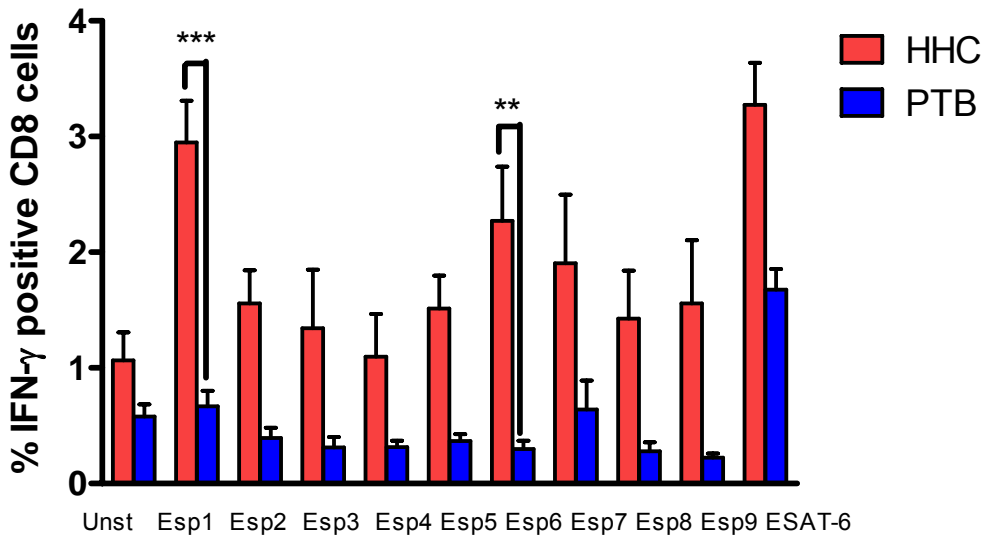
Fig.16: Intracellular IFN- γ response to ESAT-6 overlapping peptides in CD4 cells



Overlapping peptides of ESAT-6

Statistical analysis between groups was done by one way ANOVA. Significance between groups was determined by Bonferroni's post test. (** $p < 0.01$, *** $p < 0.001$)

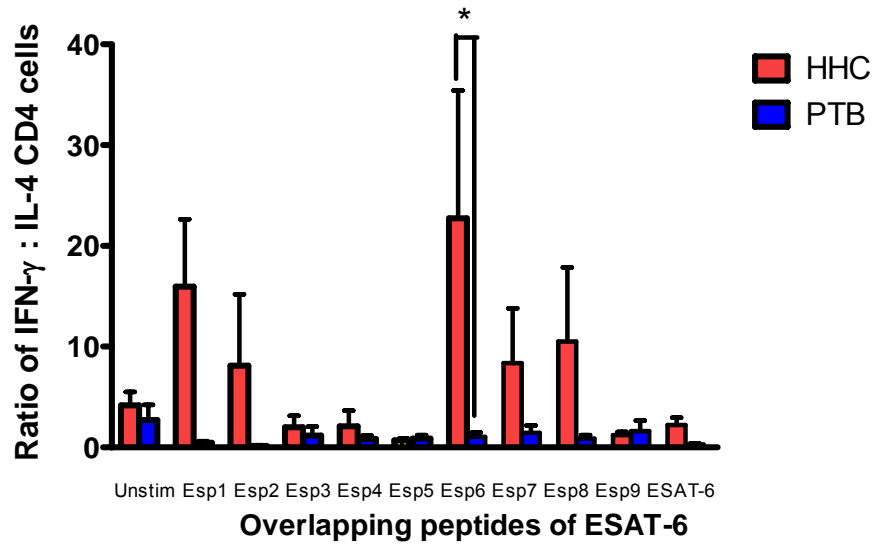
Fig.17: Intracellular IFN- γ response to ESAT-6 overlapping peptides in CD8 cells



Overlapping peptides of ESAT-6

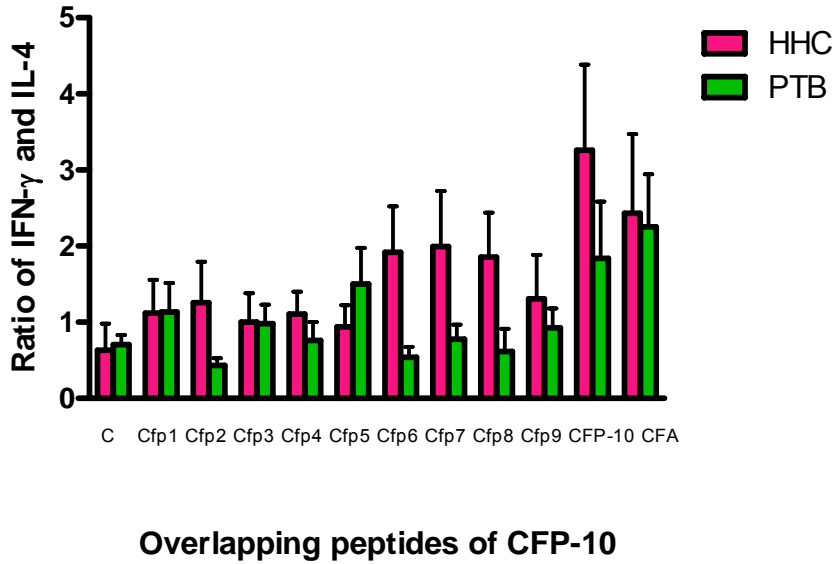
Statistical analysis between groups was done by one way ANOVA. Significance between groups was determined by Bonferroni's post test. (** $p < 0.01$, *** $p < 0.001$)

Fig.18: IFN- γ /IL-4 ratio to overlapping peptides of ESAT-6 in CD4 cells



Statistical analysis between groups was done by one way ANOVA. Significance between groups was determined by Bonferroni's post test. (*p<0.05)

Fig.19: IFN- γ /IL-4 ratio to overlapping peptides of CFP-10 in CD4 cells



[Contact person: Dr. Alamelu Raja (E-mail ID: alamelur@trcchennai.in)]

Innate immunity in HIV infection

Background

HIV-infected individuals are subjected to various immune disorders, particularly when co-infected with TB disease. Natural Killer (NK) cells are characterized by potent cytotoxic activity against tumors, virus-infected cells and intracellular parasites without prior sensitization and major histocompatibility complex restriction. Defective NK cell functions are among the various immunological abnormalities in HIV infection and are known to be partially restored *in vitro* by IL-2 and IL-12. IL-15 shares receptor and several biological properties with IL-2. A study was undertaken to look at the effect of stimulants such as *M. tuberculosis* and cytokines IL-2, IL-12 and IL-15 on the expression of various cytokines. NK cytotoxicity upon *in vitro* stimulation was also studied.

Aim

- To demonstrate NK cell mediated innate immune response in HIV-TB

Methods

Study groups included normal healthy subjects (NHS n=15), patients with pulmonary TB (TB n=15), HIV positive subjects without TB (HIV n=15) and HIV positive patients with TB (HIV-TB n=15).

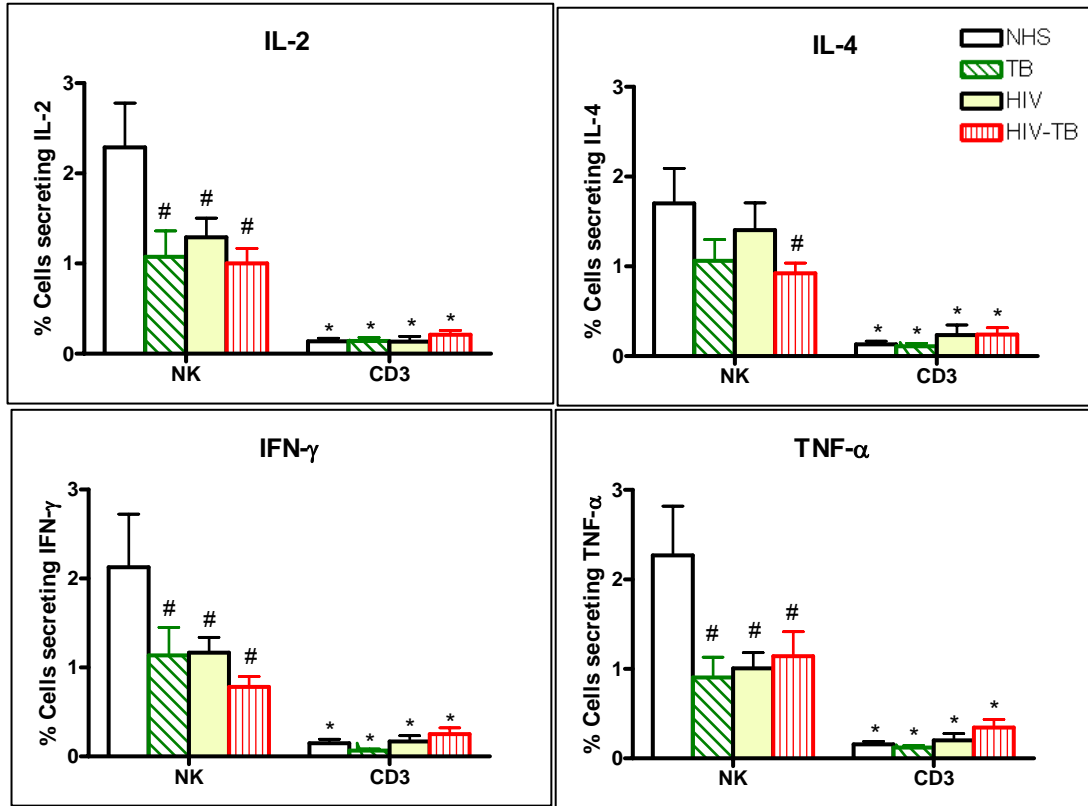
In vitro intracellular cytokine secretion (IL-2, IL-4, IFN- γ and TNF- α), response of NK cells to *M. tuberculosis* H₃₇Rv and stimulatory cytokines (IL-15, IL-15+IL-12, IL-15+IL-2) was studied using flow cytometry. The cytotoxic activity of NK cells against tumor cell lines (K562) after *in vitro* stimulation was assessed by a flow cytometry based assay.

Results

Cytokine response of NK cells to *M. tuberculosis* H₃₇Rv and stimulatory cytokines in two of the study groups, NHS and TB, were reported in Annual Report 2006-07. Results for all four groups are presented here. Basal cytokine expression pattern of lymphocytes among various groups is presented in (Fig.20). The expression of Th1 cytokines IL-2, IFN- γ and TNF- α was decreased significantly in TB, HIV and HIV-TB groups ($p < 0.05$), compared with NHS. No such difference in cytokine pattern was observed in CD3+ cells.

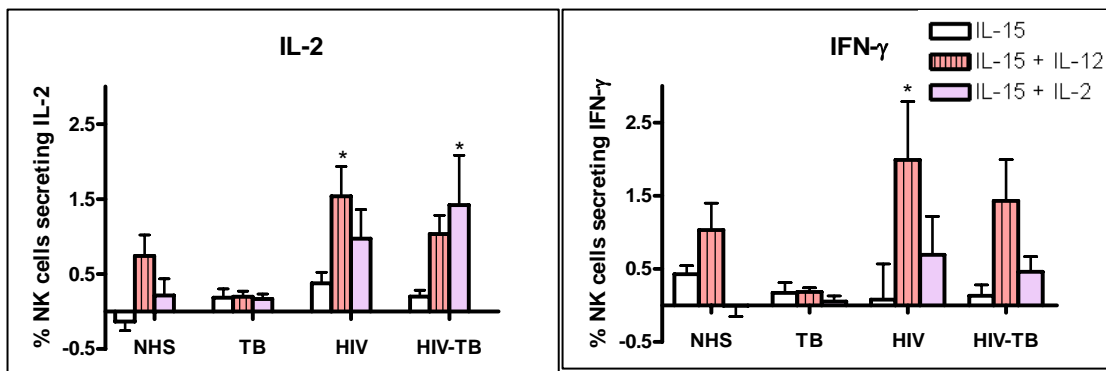
The secretion of Th2 cytokine (IL-4) by NK cells was decreased only in HIV-TB patients compared to NHS. When we compared cytokine expression between NK and CD3 cells, the expression of Th1 and Th2 cytokines were elevated in NK cells for all groups ($p < 0.05$). With *M. tuberculosis* stimulation, we observed increased Th1 cytokine in NHS compared to basal response. No significant changes were observed with other groups (data not shown). Upon stimulation with IL-15+IL-12, we found increased IL-2 and IFN- γ secreting NK cells in HIV and HIV-TB groups ($p < 0.05$) compared to NHS (Fig.21). Maximal NK cytotoxicity ($p < 0.05$) was observed in the presence of IL-15+IL-12 combination in NHS, TB, HIV and HIV-TB (Fig.22). At 50:1 effector/target concentration, the mean increase in NK cytotoxicity upon stimulation with IL-15+IL-12 was 2-fold for HIV- positive subjects and 1.8 fold for HIV-TB co-infected patients (data not shown). Augmentation of Th1 cytokine secreting NK cells and NK cytotoxicity after stimulation with IL-15+IL-12 in HIV-positive subjects and HIV-TB co-infected patients suggests its potential in improving NK cell function.

Fig.20: Basal cytokine expression pattern of lymphocytes among various groups



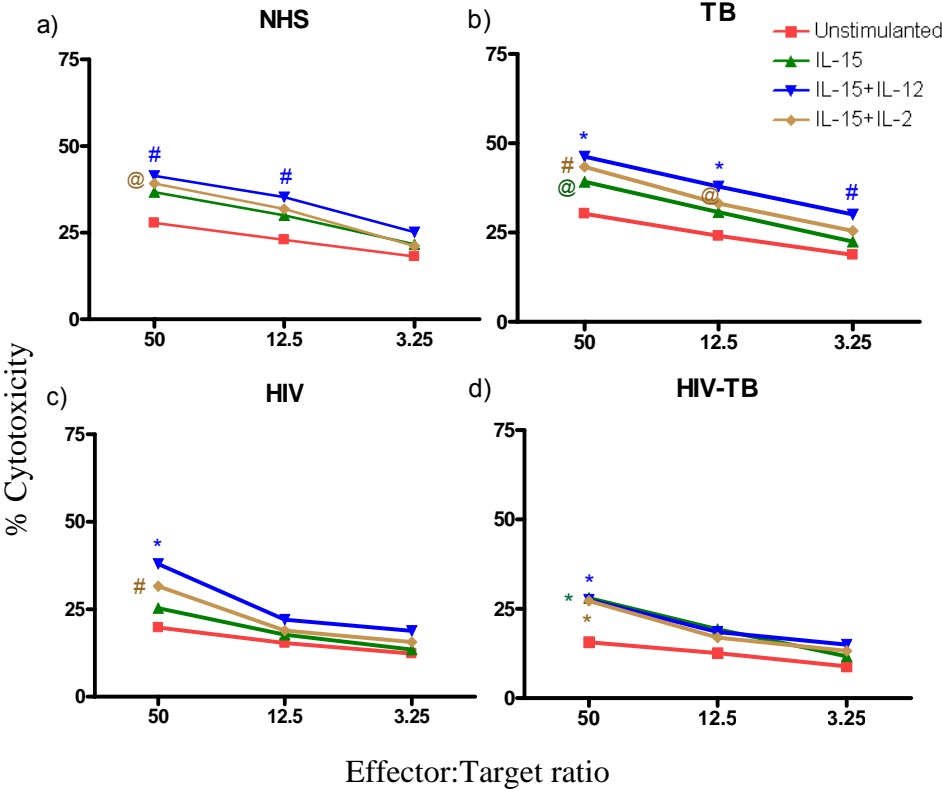
Data are represented as mean + SEM of 15 subjects. # represents the significance ($p < 0.05$) when compared with NHS within one type of lymphocytes. * refers the significance ($p < 0.05$) where the comparisons are between NK cells and CD3+ cells

Fig.21: NK cell response upon *in vitro* stimulation with cytokines



Values are represented as mean + SEM of 15 subjects. All the comparisons are with IL-15 stimulant. * refers to $p < 0.05$

Fig.22: NK cytotoxic response to individual groups



Values are represented as mean of 15 subjects. All the comparisons are with unstimulated culture. @ represents p < 0.05, # indicates p < 0.01, * refers to p < 0.001

[Contact person: Dr. Alamelu Raja (E-mail ID: 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 reactivation of latent TB infection. Because of the associated higher risk of mortality, tests that detect *M. tuberculosis* infection and disease at early stages are needed to initiate therapy.

Aim

- To assess the sensitivity of interferon gamma releasing assay (IGRA) for active TB diagnosis in patients with or without HIV infection

Methods

The study subjects are as follows:

- TB (N = 139)
- HIV-TB (N = 105)

Tuberculin skin testing (TST) was performed and read as per standard procedures, using 2 TU of PPD RT23 (Statens Serum Institut, Denmark) and reading was taken 48-72 hrs post testing. Whole blood IGRA was done by using Quantiferon TB Gold kit (QFT-G) (Cellestis, Victoria, Australia) as per the manufacturer's instructions.

Results

Sensitivity of IGRA in active TB patients

In last year's Annual Report (2006-2007), it was reported that the levels of IFN- γ have been measured in 139 patients. 0.35 IU/ml was set as cut-off value as described by the manufacturer. Among the 139 PTB patients, 124 were positive and 11 were negative for QFT-G. The remaining four patients showed indeterminate or invalid results. All the indeterminate results were due to poor response to mitogen. The four invalid results were excluded for further sensitivity analysis.

IGRA showed sensitivity of 92% in both smear positive as well as negative cases. The percentage of positivity did not vary between smear positive and negative cases. The sensitivity was consistently high even in cavitating smear positive subjects. It showed that QFT-G has the ability to identify even severe TB cases.

Among the 139 study subjects, both TST and QFT-G results were available for 89 subjects. Among them, 53 and 65 subjects showed an induration >15mm and >10mm respectively. In this study, we used 10mm cut-off value to calculate the sensitivity of TST; hence it yielded a sensitivity of 73%. In head to head comparison with QFT-G, TST showed poor agreement with the 'k' value of 0.077 (95 CI; 0.107–0.262) (table 21). However, the induration of TST and level of IFN- γ was significantly correlated in QFT-G and TST positive subjects (Fig.23). This study strongly showed that IGRA is highly sensitive and superior to TST in detecting active PTB cases. Hence, it may be used especially in smear negative cases, for whom new diagnostic methods are actually needed.

Sensitivity of QFT-G and TST in HIV-TB patients

A total of 105 HIV-TB subjects were recruited for this study. The demographic profile of the study subjects is given in table 22. Of the 105 subjects tested, 68 (65%) were positive and 19 (18%) were negative for QFT-G (table 23). The remaining 18 (17%) subjects showed indeterminate results. All the indeterminate or invalid results were due to poor response to PHA. With exclusion of indeterminate results, QFT-G showed a sensitivity of 78%. Among the 53 culture positive samples, with the 11 indeterminate and 37 positive results, QFT-G showed 88% sensitivity. The sensitivity of QFT-G was consistently high in both PTB (73%) and extrapulmonary tuberculosis (EPTB) (81%) subjects.

The median total lymphocyte, CD3, CD4 and CD8 counts were significantly lower in indeterminate cases when compared to QFT-G positives ($p=0.001$, $p=0.003$, $p=0.042$ and $p=0.022$ respectively) (Fig.24). None of them significantly differed between QFT-G positive and negative cases.

Of the 102 TST study results available, 34 showed ≥ 5 mm induration for TST; hence it yielded a sensitivity of 34%. The median of total lymphocytes, CD3 cells and CD4 cell counts were significantly lower in TST negative subjects when compared to TST positive subjects.

The results clearly evidenced that the sensitivity of QFT-G was retained in HIV-TB cases and the sensitivity was also not depressed by low CD4 counts. While comparing the QFT-G and TST, the latter showed low sensitivity.

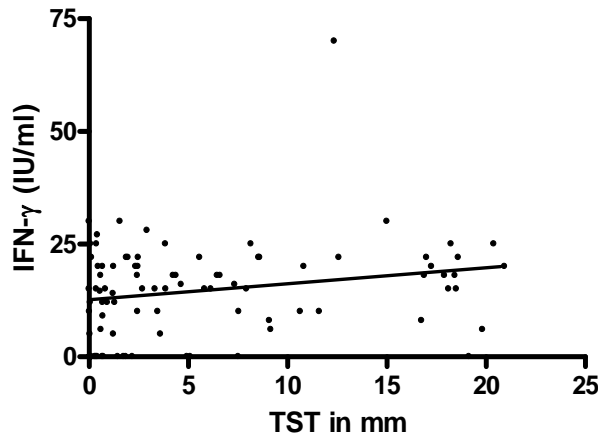
Recruitment of study subjects to the different groups is in progress.

Table 21: Agreement between TST and QFT-G

		QFT-G		
		Positive	Negative	Total
TST	Positive	58	4	62
	Negative	21	3	24
	Total	79	7	86

Agreement between the two tests was calculated using kappa statistics.
Kappa value 0.077

Fig.23: Correlation between TST induration and magnitude of IFN- γ levels



The correlation between the magnitude of IFN- γ and TST induration was assessed by Pearson correlation analysis. $R = 0.27$, $P = 0.01$

Table 22: Demographic and baseline parameters of the 105 HIV-TB subjects

Sex, number (%)	Male	78 (74%)
	Female	27 (26%)
Age, Median in years (Range; IQR)		36 (18-63; 25, 36)
BMI, kg/m ² (Range; IQR)		19 (13-31; 17,21)
HIV strain, Number (%)	HIV-I	89 (89%)
	HIV-I&II	11 (11%)
TB types, Number (%)	PTB	63 (60%)
	EPTB	39 (37%)
Smear result, Number (%)	Positives	47 (45%)
	Negatives	58 (55%)
Culture result, Number (%)	Positives	53 (50%)
	Negatives	52 (50%)
CD4, Median (Range; IQR) (For available 81 subjects)		116 (11-2062; 48, 209)
CD4 count, Number (%)	<100	38 (47%)
	100-199	21 (26%)
	>200	22 (27%)

BMI - Body mass index, IQR – Inter quartile range

Table 23: Sensitivity of QFT-G in HIV-TB subjects

Groups	QFT-G			
	Pos (%)	Neg (%)	Ind (%)	Sen (%)
Overall (N=105)	68 (65)	19 (18)	18 (17)	78
Smear positive (N=47)	32 (68)	4 (9)	11 (23)	89
Smear negative (N=58)	37 (64)	14 (24)	7 (12)	73
Culture positive (N=53)	37 (70)	5 (9)	11 (21)	88
Culture negative (N=52)	32 (62)	13 (25)	7 (13)	71
PTB (N=66)	39 (59)	13 (20)	14 (21)	75
EPTB (N=39)	30 (77)	5 (13)	4 (10)	86

QFT-G - Quantiferon TB Gold

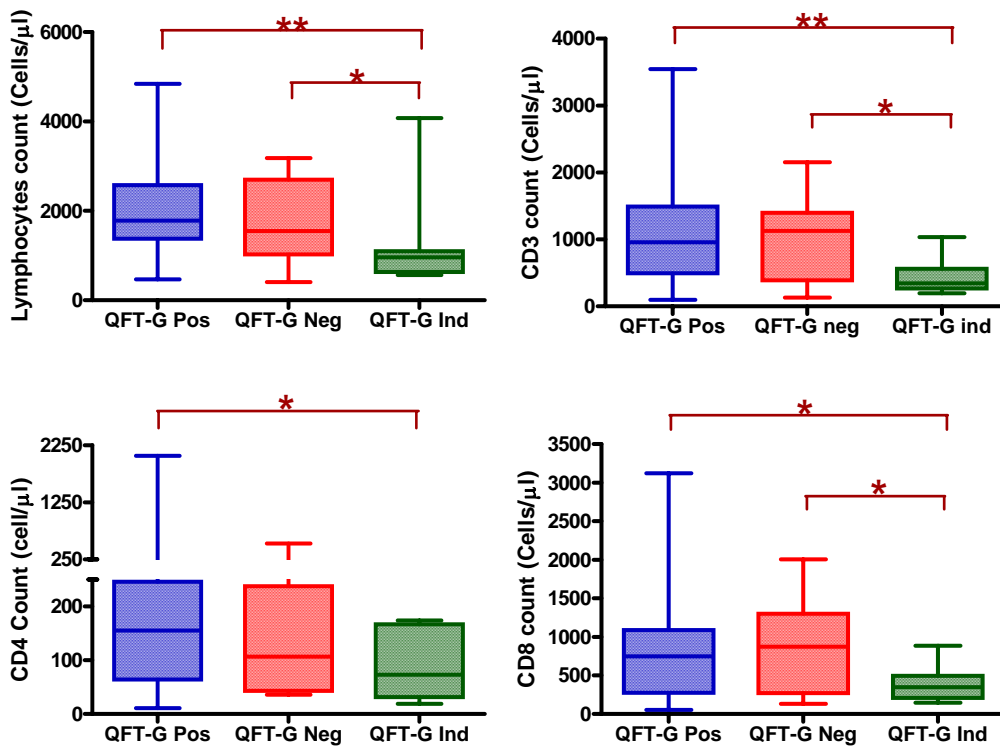
Pos - Positive (Secretion of IFN- γ in response to TB antigens was >0.35 IU/ml)

Neg - Negative (Secretion of IFN- γ in response to TB antigens was <0.35 IU/ml and the mitogen response was >0.5 IU/ml)

Ind - Indeterminate (Secretion of IFN- γ in response to TB antigens was <0.35 IU/ml and the mitogen response was <0.5 IU/ml)

Sen - Sensitivity [Number of positives/(Total subjects - Number of indeterminates)]

Fig.24: Level of total lymphocyte & T-cell count in QFT-G positive, negative & indeterminate subjects



The difference between groups was assessed using Mann-Whitney U test. * P<0.05 ** P<0.01
 QFT-G - Quantiferon TB Gold, Pos – positive, Neg – negative, Ind - indeterminate

[Contact person: Dr. Alamelu Raja (E-mail ID: alamelur@trchennai.in)]

Differential upregulation of chemokine receptors on CD56⁺ NK-cells and their transmigration to the site of infection in tuberculous pleuritis

Background

Chemokines and their receptors orchestrate the leukocyte recruitment and confer immunity during *M. tuberculosis* infection. The immunoregulatory and cytotoxic activity of NK-cells are essential at the site of infection during tuberculous pleurisy.

Aim

To assess the frequency, subtypes, expression of phenotype markers and chemokine receptors on NK-cells in tuberculous and non tuberculous pleural fluid (PF).

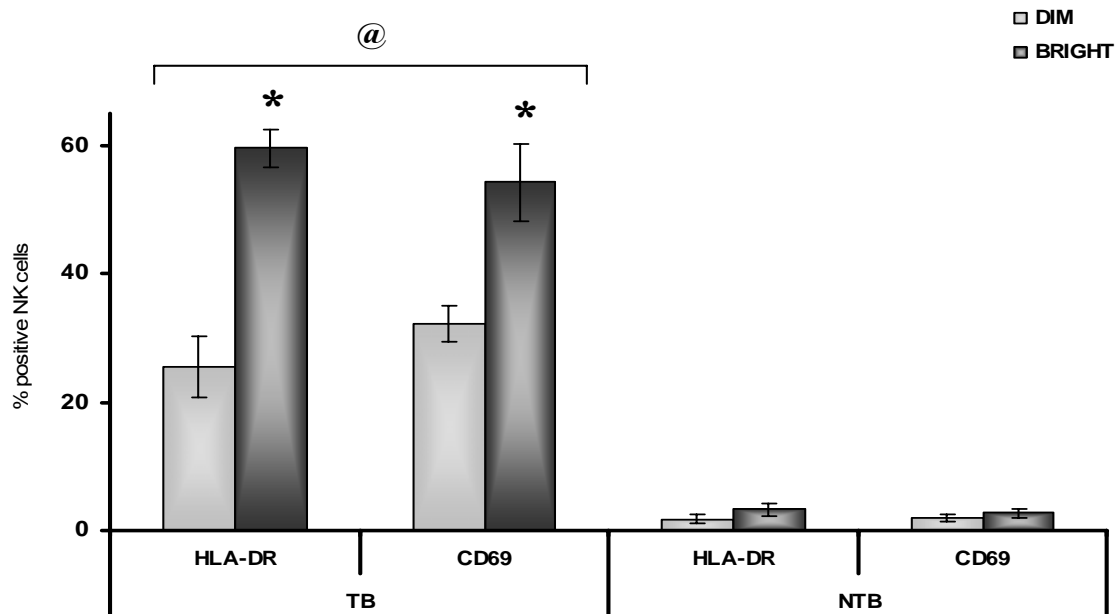
Methods

Pleural fluid mononuclear cells from TB and NTB groups were stained with CD3-FITC and CD16/56-PE antibodies to identify the NK-cell population in the lymphocyte gate by flow cytometry analysis. The NK-cell subsets were characterized for the surface expression of CD69, HLA-DR, TLR (TLR-2, TLR-4 and TLR-9) α - and β - chemokine receptors (CXCR2, CXCR3, CCR1, CCR2, CCR5 and CCR7) using CCR5 and TLR9-FITC, CCR1, CCR2, CCR7, TLR-2 and TLR4-PE, CXCR2-APC labeled mouse anti-human antibodies.

Results

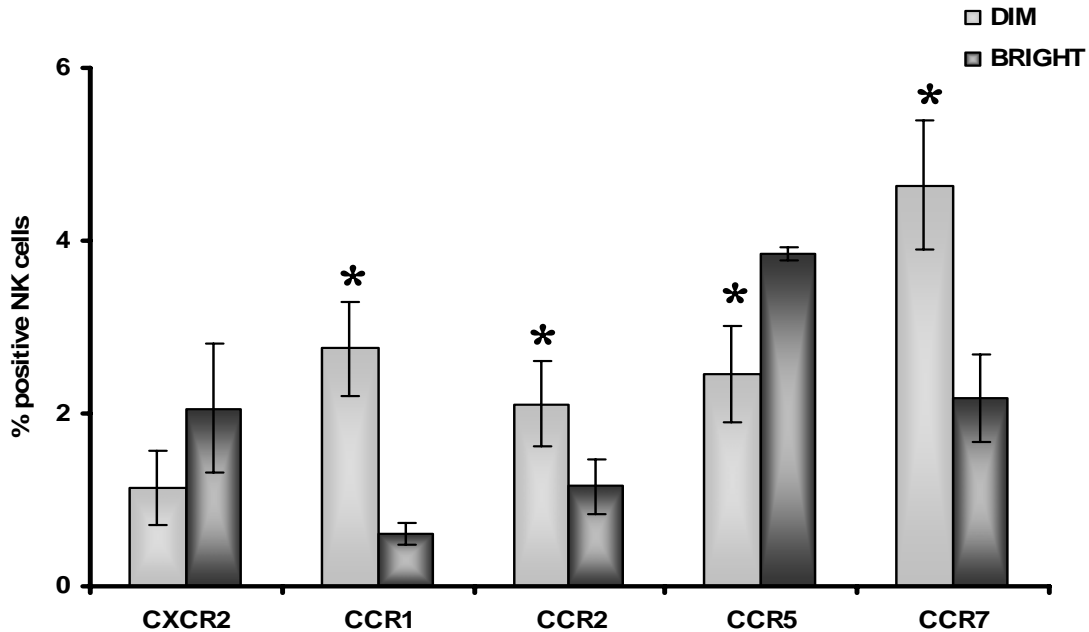
A significant decrease in CD56dim with no change in CD56bright NK-cells was observed in the TB group (Fig.25). Significantly increased expression of chemokine receptors CCR1, CCR2 and CCR7 on CD56bright and CCR5 on CD56dim NK-cells was also observed in the TB group (Fig.26).

Fig.25: Phenotype characterization of pleural fluid NK-cell subsets in TB (N=38) & NTB (N=24) patients



Cells were stained dually and analyzed by flow cytometry for co-expression of HLA-DR and CD69 on the subsets of NK-cells in both the study groups. Results are expressed as mean values \pm standard error of mean (SEM). * @ $P < 0.05$ was considered to be significant using independent student's t-test and Mann-Whitney U test for comparison between groups. An isotype control was used to set the fluorescent compensation and to minimize the overlap of fluorochrome signals or background staining.

Fig.26: Differential expression of chemokine receptors on the subsets of NK-cells in TB



Co-expression analysis for chemokine receptors (CXCR2, CCR1, CCR2, CCR5 and CCR7) on TB and NTB PF NK-cells was performed. Results are expressed as mean values \pm standard error of mean (SEM). * $P < 0.05$ was considered to be significant using independent student's t-test. An isotype control was used to set the fluorescent compensation and to minimize the overlap of fluorochrome signals or background staining.

Conclusion

The study suggests that CD56bright NK-cells may recognize *M.tuberculosis* directly using toll-like receptors (TLRs), HLA-DR and CD69. In addition, CC chemokines induce activation signals mediating differential NK-cell migration to the site. Thus NK-cells act as first direct sensors and effectors in mycobacterial infection.

[Contact person: Dr. Sulochana D Das (E-mail ID: dsulochana@trchennai.in)]

Role of dendritic cells in mycobacterial immunity - Retarded migration of *M. tuberculosis* infected dendritic cells

Background

Dendritic cells (DCs) act as sentinels against pathogens in the host immune system. Their role in the initiation and regulation of the T lymphocyte response towards *M. tuberculosis* is fundamental during infection. Firstly, they are able to reach the site of infection and secondly, after recognizing the pathogen, they are activated and migrate towards lymph nodes to activate naive T lymphocytes. The movement of DC is therefore crucial in the immunological functions.

Aim

- To investigate *in vitro*, the effect of virulent *M. tuberculosis* (H₃₇Rv) and prevalent clinical isolates of *M. tuberculosis* (S7 and S10) on the migration and chemotactic activity of DCs derived from human monocytes

Methods

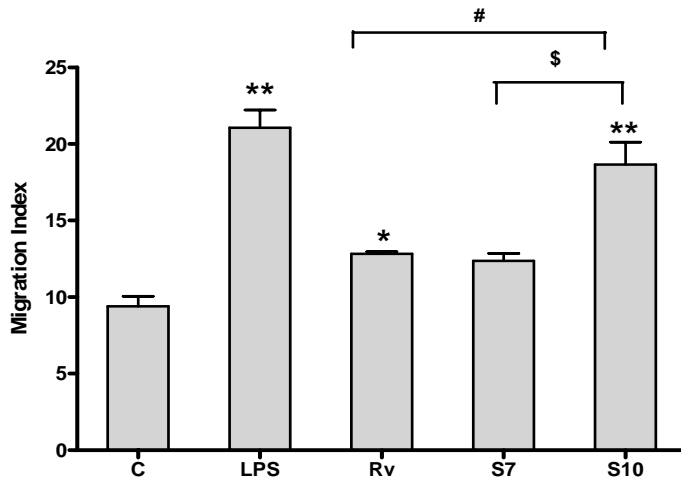
Peripheral blood mononuclear cells were isolated from the blood of healthy individuals. Monocytes were purified by using anti-CD14 conjugated magnetic beads. Monocyte derived DCs (MoDCs) were generated by culturing CD14⁺ cells with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 for 5 days. On day 5, the MoDCs were either stimulated with LPS or infected with different *M.tuberculosis* strains. After 24 hours, DCs were harvested and analyzed using fluorescent activated cell sorter (FACS) for chemokine receptors (CCR5 & CCR7). ELISA for chemokines (IL-8, IP-10, MCP-1, MIP-1 α) was performed in the culture supernatants. The infected MoDCs were subjected to chemotaxis assay for studying the migration activity using recombinant CCL21 (secondary lymphoid chemokine – SLC) and the chemotactic activity of infected MoDCs was performed using T lymphocytes.

Results

The levels of chemokines that were undertaken in this study were low in *M. tuberculosis* infected MoDCs. The chemotaxis assay demonstrated suppressed migration of MoDCs after infection (Fig.27). Also the chemotaxis of T-cells

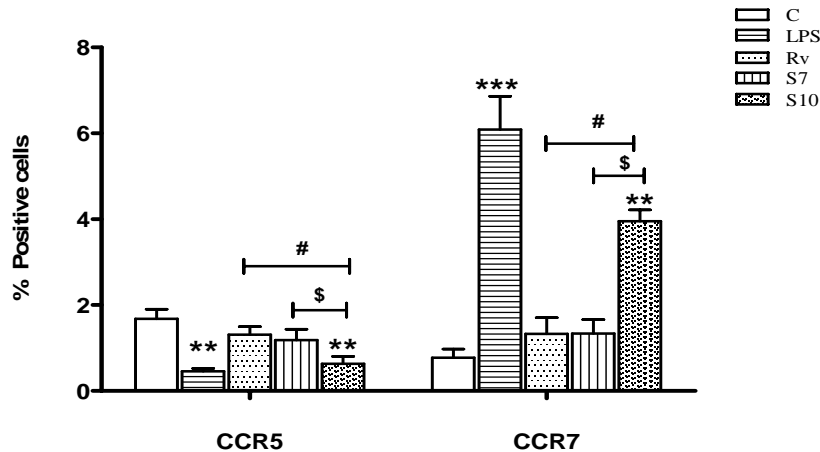
against infected MoDCs and supernatants was lesser with Rv and S7 infection. The expression of CCR5 was not down regulated in *M. tuberculosis* infected MoDCs as compared to LPS stimulated MoDCs, while CCR7 was not significantly upregulated with Rv and S7 infected MoDCs (Fig.28).

Fig.27: Migration of infected MoDCs to recombinant SLC



Chemotaxis of *M. tuberculosis* infected MoDCs to recombinant CCL21. The results of three independent experiments are depicted as mean \pm SEM. The statistical significance is shown as * compared to uninfected control, # compared to Rv and \$ compared to S7

Fig.28: Expression of chemokine receptor on infected MoDCs



Surface expression of CCR5 and CCR7 on MoDCs is shown (a) after infection with different strains of *M. tuberculosis*. Data represented as mean \pm SEM obtained from ten different experiments. The statistical significance is shown as * compared to uninfected control, # compared to Rv and \$ compared to S7.

Conclusion

The study findings suggest that the modulated migration of DC and its cell trafficking ability may be a potent mechanism used by *M. tuberculosis* to paralyze the early immune response of the host.

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Development of mice model of latent TB (Funded by the Department of Biotechnology)

Background

This is a collaborative project between TRC and University of Delhi, south Campus. The aim of this project is to study the immunomodulation of latent TB by recombinant strains of BCG over-expressing various *M. tuberculosis* antigens. At TRC, the pathological changes pertaining to the disease and immunomodulation are being assessed using conventional and immuno histochemical procedures. During the period under review, the basic model for establishing latent TB using aerosol infection was standardized and the study is in progress using recombinant BCG strains.

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Protein engineering of self-assembly systems for applications in nanoscience and nanotechnology (Funded by the Department of Biotechnology)

This is a collaborative project between TRC, Madurai Kamaraj University and Anna University. The aim of this project is to study the expression of HIV-1C gp41 epitopes on two self-assembly systems *viz.* the outer membrane porins of *Salmonella typhi* and the coat protein of cardamom mosaic virus. This will be used to display a highly conserved epitope from the GP41 of human immunodeficiency virus 1. The HIV-1 epitope (aa 731-752 of GP41) harbors a neutralization domain. Expression of this epitope in multiple copies on a self-assembly system is likely to lead to an efficient vaccine against HIV. These chimeric systems that form nanoparticles will be biologically characterized. The

production and physico chemical characterization of these self assembly systems will be performed by the two partnering institutions. TRC will be assessing the biological effects by studying the profile of antibodies and interaction with lymphocytes in HIV-infected individuals reacting with these self assembly systems. During the period under review, the initial characterization of a system using cardamom mosaic virus coat protein has been carried out.

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