NATIONAL INSTITUTE FOR RESEARCH IN TUBERCULOSIS

Research Activities

April 2011 – March 2012

CONTENTS

	Page No.
Preface	3
Committees	4
Abbreviations	10
Research Activities	
1. Department of Clinical Research	12
HIV Vaccine Trial	20
Socio-behavioral studies	21
Laboratory studies	25
2. Department of Bacteriology	43
3. Department of Immunology	48
4. Department of Statistics	94
5. Biomedical Informatics	107
6. Epidemiology	110
7. Electronic Data Processing	114
8. International Centre of Excellence in Research	116
9. NIRT-Institutional Ethics Committee Activities	125
10. Library & Information Centre	126
Appendices	
Publications	127
Awards/Honours	139
Workshops/ Conferences, etc., organized	139
Ph.D. Scholars	140
Staff list	144

PREFACE

This edition of the Annual Report of the Institute highlights the efforts undertaken to provide high quality research support for tuberculosis (TB) control. The priority has been, as before, to identify regimens that will be of use in national as well as global programmes for the control of TB and HIV-TB. During the year under review, our efforts to evolve patient friendly and cost effective regimens for the treatment of TB continued. This included studies to shorten the duration of treatment regimens for TB, treatment of HIV/TB co-infection and management of MDR-TB. Closely linked to the evaluation of chemotherapy is the study of behavioral aspects of patients and health systems.

The Epidemiology division is carrying out studies to identify risk factors such as use of tobacco, in populations where TB is endemic.

The upgraded Bacteriology department provides expert services of not only all forms of TB, but also in the field of MDR and XDR-TB. The LPA facility for rapid diagnosis of MDR-TB obtained accreditation. Other important research activities of the institute include laboratory studies in Bacteriology, Immunology, Clinical Pharmacology, Molecular Epidemiology and Molecular Virology.

The Institute continues to be recognized for its expertise in the field of TB Research and Training for which it is recognized as a WHO Collaborating Centre and also as an International Centre of Excellence in Research (ICER) by the National Institutes of Health which has funded a laboratory at NIRT through an intramural grant.

The studies carried out by this Institute fully support the national and international programmes for the control of TB and HIV and the members of the Institute play significant roles both scientifically and technically in strengthening TB control globally and nationally.

I place before you this Annual Report that represents the combined efforts of all the staff members and invite your valuable suggestions to help us improve our research efforts.

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ABBREVIATIONS

Acquired rifampicin resistance (ARR)
Alcohol use disorders (AUDs)
Allelic exchange substrate (AES)
Anti-retroviral treatment (ART)
Anti-TB treatment (ATT)
Antigen presenting cells (APC)
Body mass index (BMI)
Bovine serum albumin (BSA)
Complimentary DNA – (Cdna)
Culture filtrate antigen (CFA)
Cytolytic T lymphocytes (CTL)
Cycloserine (Cs)
Data and Safety Monitoring Board (DSMB)
Designated microscopy centres (DMC)
Drug-resistant (DR)
Drug resistance surveillance (DRS)
Drug susceptibility testing (DST)
Efavirenz (EFV)
Ethambutol (EMB)
Ethionamide (Eth)
Extracellular matrix (ECM)
Fetal calf serum (FCS)
Fourier transform infrared spectroscopy (FTIR)
Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)
Healthy household contacts (HHC)
Healthy contacts (HC)
High performance liquid chromatography (HPLC)
Immune reconstitution inflammatory syndrome (IRIS)
Inducible protein (IP)
Interleukin (IL)
Isoniazid (INH)
Kirchner's liquid medium (KLM)
Layer chromatography (TLC)
Levofloxacin (LFX)
Liquid chromatography mass spectrometry (LCMS)
Male sex workers (MLSWs)
Matrix metallo proteinases (MMPs)
Men having sex with men (MSM)
Mitogen-Activated Protein Kinases (MAPKs)
Monocyte chemoattractant protein-1 (MCP-1)
Monokine induced by interferon- γ (MIG)
Mothers living with HIV (MLH)
Moxifloxacin (MFX)
Multi-drug resistant TB (MDR-TB)

Mycobacteriophage genome database (MGDB)
National Institute for Research in Tuberculosis (NIRT)
Natural killer (NK)
New sputum smear positive cases (NSP)
Nevirapine (NVP)
Nonnucleoside reverse transcriptase inhibitor (NNRTI)
Nucleoside reverse transcriptase inhibitors (NRTIs)
Nuclear magnetic resonance (NMR)
Ofloxacin (OFX)
Pleural mesothelial cells (PMC)
Population proportion to size (PPS)
Polymerase chain reaction (PCR)
Protease inhibitors (PIs)
Proximal support vector machines (PSVM)
Pulmonary TB (PTB)
Pyrazinamide (PZA)
Randomized controlled trial (RCT)
Real time PCR (RT-PCR)
Restriction fragment length polymorphism (RFLP)
Re-treatment (RT)
Revised National TB control program (RNTCP)
Rifampicin (RMP)
Rifabutin (RBT)
Ritonavir (RTV)
Serine/threonine protein kinases (STPKs)
Sexually transmitted infections (STIs)
Short course chemotherapy (SCC)
State drug stores (SDS)
Structural risk minimization (SRM)
Support vector machines (SVM)
TB Unit (TU)
TB lymphadenitis (TBL)
Thin layer chromatography (TLC)
Tibotec medicinal research compound (TMC)
Tissue-inhibitors of metalloproteinases (TIMPs)
Tuberculosis (TB)
Tuberculin skin test (TST)
Quantiferon TB-Gold in tube (QFT-IT)
Viral load (VL)
World Health Organization (WHO)

DEPARTMENT OF CLINICAL RESEARCH

Studies Completed:

(i) A Phase II, placebo-controlled, double-blind, randomized trial to evaluate the anti-bacterial activity, safety and tolerability of Tibotec medicinal compound (TMC) 207 in participants with sputum smear-positive pulmonary infection with multi-drug resistant *M. tuberculosis*

(Principal Investigator: Dr. Aleyamma Thomas; Funding: Tibotec BVBA)

Background: Development of new TB drugs is a priority. Tibotec medicinal compound (TMC) 207 is a methoxyquinoline with potential value in combination treatment of TB. It has a unique structure with high *in vitro* activity against wild-type and drug-resistant (DR) strains of *M.tuberculosis*. TMC207 was found to be generally safe and well tolerated in phase I studies. In the exploratory Phase I of the trial completed in South Africa, results of the interim analysis showed that addition of TMC207 to a 5-drug multi-drug resistant TB (MDR-TB) regimen resulted in significantly shorter time to culture conversion compared to placebo.

Aim: To demonstrate the anti-bacterial activity of TMC207 compared to placebo when added for 24 weeks to a background regimen in participants with newly diagnosed sputum smear-positive pulmonary MDR-TB infection

Methods:

Study design: National Institute for Research in Tuberculosis (NIRT) was involved in Phase II of the trial. This was a stratified, randomized, double-blind, multi-centric, placebo-controlled Phase II trial.

TMC 207 was given at 400mg dosage once a day for first two weeks followed by 200 mg thrice a week for the next 22 weeks along with the background regimen consisting of kanamycin, ethionamide (Eth), pyrazinamide (PZA), ofloxacin (OFX) and cycloserine (Cs) in doses based on body weight. The patients were monitored weekly for two months, fortnightly for four months and monthly for 18 months.

Results: The study was initiated in August 2009. A total of 3 patients were enrolled, among whom one was a diabetic. All the patients had culture conversion. The patients were followed up for two years; this was completed in March 2012. A total of 150 patients were admitted to this study from different centres. Analysis of the data is in progress.

Studies in progress:

(i) A randomized controlled clinical trial comparing daily vs. intermittent 6 – month short course chemotherapy in reducing failures & emergence of Acquired Rifampicin Resistance in patients with HIV and pulmonary TB (CTRI Registration No: 476/09, NCT No: 933790)

(Principal Investigator: Dr. G. Narendran, Co-PI: Dr. Soumya Swaminathan; Funding Agency: Intramural or United States Agency for International Development (USAID) through WHO Model DOTS Project)

This study comparing daily vs. intermittent therapy of anti-TB treatment (ATT) is a unique opportunity to compare various aspects of TB outcome in HIV-pulmonary TB (PTB) such as sputum conversion, immune reconstitution inflammatory syndrome (IRIS), emergence of acquired rifampicin resistance (ARR), radiological improvement and toxicity profile. The three regimens are: (i) daily regimen ($2EHRZ_7/4HR_7$), (ii) partly intermittent ($2EHRZ_7/4HR_3$) and (iii) a fully intermittent regimen ($2EHRZ_3$ /$4HR_3$), given for 6 months duration and followed up for a further period of one year.

As on 01.03.12, 125 patients (33 from Madurai and 92 from Chennai) have been allocated to the three regimens randomly (Daily - 41, Part daily - 44, Intermittent - 40). Mean age and weight of the study participants were 39 years and 43 kg respectively. The median CD4 cell counts was 135 cells /mm3 (IQR-70-252) and the median viral load (VL) being 211000 copies/ml (IQR – 39450 -592913); most patients were in the advanced stage of HIV. At present, all the regimens are equally efficacious and the study is ongoing.

(ii) Study to evaluate the effect of Physician's advice in quitting smoking in HIV and TB patients in south India - A pilot study

(Principal Investigator: Dr. S. Ramesh Kumar (Funding: Fogarty grant, Miriam Hospital, Brown University, USA)

(No.: CTRI / 2009 / 091 / 000962 dt.14.12.2009)

Background: The burden of HIV and TB in India is high. The association of TB and smoking is evident. Smoking in HIV poses additional risks. Smoking cessation initiation by Physician's advice has been shown to be useful in previous studies.

Aims: (i) to determine the efficacy of Physician's advice using "modified 5 A" strategy in quitting smoking in patients with HIV and patients with TB and (ii) to compare the effectiveness of Physician's advice with administering abrochure containing smoking cessation information and a counselor's counseling to brochure and counselor counseling alone in quit rate in patients with HIV and patients with TB

Sample size: HIV patients (smokers) = 80;

TB patients (smokers) = 80

Methods: Patients with TB or HIV with history of current smoking referred to the clinic will be randomized to receive Group A (Physician's advice + Counselors counseling + Brochure / educative material) or Group B (Counselors counseling + Brochure / educative material) strategy of smoking cessation, stratified based on Nicotine dependence assessed by using Fagerstrom dependence scale. In Group A, in addition to educating using brochures and a standard counseling by counselor (strategy in Group B), Physician's advice using 'modified 5 A' strategy will be systematically approached in the five standard steps namely "Ask, Advise, Assess, Assist and Arrange". In addition, the physician will deliver a brief structured advice to subject and his/her family member. This is the change in the 'Assist' step of the standard "5 A" approach and hence we call the strategy as 'modified 5 A' approach.

Recruitment: Patient recruitment to this study was initiated in March 2010. At the end of March 2012, recruitment was completed; 160 subjects (80 TB and 80

HIV) were recruited. Among them 80 each were allocated to Groups A (Physician's arm) and B. These patients are being followed up.

The study has been registered in Clinical trials registry of India.

(iii) Randomized Clinical Trial to study the efficacy and tolerability of 3- and 4-month regimens containing moxifloxacin in the treatment of patients with sputum positive pulmonary TB

(Principal Investigator: Dr.M.S. Jawahar)

Shortening the duration of TB treatment is a research priority. To address this issue, the NIRT is investigating the efficacy and safety of 3- and 4-month regimens in comparison with that of the standard 6-month regimen for the treatment of patients with sputum positive pulmonary TB. In this randomized clinical trial, the standard 4-drug TB regimen is supplemented with moxifloxacin (MFX), a fluoroquinolone with potent bactericidal and sterilising activities against *M. tuberculosis*. Patients with newly diagnosed sputum positive, HIV sero-negative pulmonary TB, resident in Chennai and Madurai are randomly allocated to 3-month or 4-month MFX, regimens, or a 6-month regimen as control. Treatment is directly observed and response to treatment is assessed by clinical evaluations and with sputum examinations. The patients are also closely monitored for adverse drug reactions which are critically documented. Patients with successful treatment outcome are followed up for 24 months with monthly evaluations for assessing recurrence of TB. The regimens are shown in table 1.

Table 1: Study regimens

Regimen	Months						Months
	1	2	3	4	5	6	
Test Reg. 1	RHZEM						3
Test Reg. 2	RHZEM		RHM				4
Test Reg. 3	RHZEM		RHM_3				4
Test Reg. 4	RHZEM		$RHEM_3$				4
Control Reg.	$RHZE_3$		RH_3				6

R – rifampicin; H – isoniazid; Z – pyrazinamide; E – ethambutol; M - moxifloxacin

Intensive phase Continuation phase

As of 31st March 2012, a total of 618 patients have been enrolled in the study. The baseline characteristics of these patients are shown in table 2.

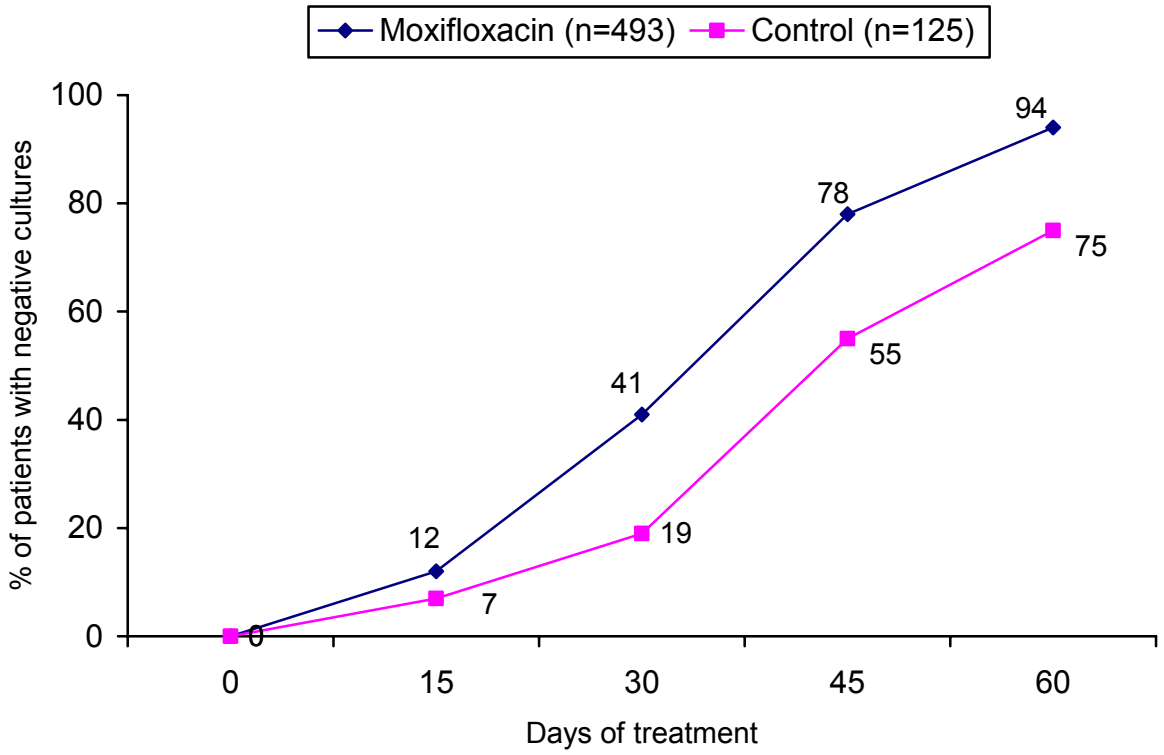
Table 2: Baseline characteristics of 618 patients enrolled in study

Regimen	Test Reg. 1 (n = 111)	Test Reg. 2 (n = 121)	Test Reg. 3 (n = 139)	Test Reg. 4 (n = 122)	Control Reg. (n = 125)	Total (n = 618)
Sex						
Male	88 (79%)	91 (75%)	105 (76%)	86 (71%)	99 (79%)	469 (76%)
Female	23 (21%)	30 (25%)	34 (24%)	36 (29%)	26 (21%)	149
Age						
<40 years	77 (69%)	73 (60%)	94 (68%)	81 (66%)	80 (64%)	405 (66%)
≥40 years	34 (31%)	48 (40%)	45 (32%)	41 (34%)	45 (36%)	213
Initial sputum culture						
1+	3 (3%)	6 (5%)	8 (6%)	4 (3%)	2 (2%)	23
2+ or 3+	108 (97%)	115 (95%)	131 (94%)	118 (96%)	123 (98%)	595 (96%)
Extent of Initial X-ray involvement (zones)						
≤ 2	24 (22%)	26 (21%)	31 (22%)	28 (23%)	25 (20%)	134
> 2	87 (78%)	95 (78%)	108 (78%)	94 (77%)	100 (80%)	484 (78%)

Sputum culture conversion with treatment

A salient finding of this study is that the proportion of patients who became sputum culture negative after the initial 2 months of treatment was significantly higher (94%) in the MFX arm (consolidated for all four test regimens) compared to the control arm (75%). This observation which was made earlier (Annual Reports 2009-2010, 2010-2011) is sustained even with the larger population. Fig. 1 illustrates the proportion of patients with positive sputum cultures at 15, 30, 45 and 60 days of treatment. This is a significant finding as it shows that patients treated with the test regimens become less infectious earlier and to a greater degree compared to those treated with the control regimen.

Fig. 1: Sputum culture conversion with treatment in 618 patients



Results at end of treatment

Table 3 describes the results at the end of treatment in 555 patients. Among patients treated with MFX regimens, 90 to 95% had all cultures negative at the end of treatment compared to 83% in the control regimen. Six patients required change of treatment for either drug toxicity or pregnancy.

Table 3: Response at the end of treatment

Regimen	Patients	Favourable	Unfavourable			Defaulted
			Bacteriological	Treatment changed	Death	
Test Reg. 1	106^	97 (91%)		2*		7
Test Reg. 2	113	107 (95%)	1	1*	1	3
Test Reg. 3	119[$]	107 (90%)	4	1*		7
Test Reg. 4	107	100 (93%)	1	1***	1	4
Control Reg.	110	91 (83%)	4	1**	2	12

^ 3 patients who received < 80% of treatment excluded

$ 1 patient who received < 80% of treatment excluded

* for jaundice

** for rifampicin induced skin lesions

*** for pregnancy

Recurrence of TB

Patients with a successful outcome at the end of treatment are being followed up for 24 months. Of 502 such patients 47 had recurrence of TB (Table 4). TB recurrence was significantly higher in Test Regimen 1 (3-month MFX regimen) compared to the 4-month MFX regimens and the control regimen. Based on this information, the Data and Safety Monitoring Board (DSMB) recommended the temporary suspension of intake to this regimen, pending a more detailed review later. Intake to the other regimens is continuing.

Table 4: Recurrence of TB according to regimen

Regimen	Patients	Recurrence of TB	Recurrence of TB / 100 Person-years
Test Reg. 1	97	18*	9.99
Test Reg. 2	107	7	3.84
Test Reg. 3	107	9	4.58
Test Reg. 4	100	7	3.51
Control Reg.	91	6	3.74

* 1 patient relapsed with TB meningitis

The Study is registered in the Clinical Trials Registry of India (CTRI) (ctri.nic.in) (PROVCTRI/2008/091/000024)

(iv) HIV associated lipodystrophy syndrome in children: Role of nutrition, anti retroviral treatment and genes

[Principal Investigators: Dr. Soumya Swaminathan, Dr. Padmapriyadarsini; Funding Agency: National Institute of Health (RO1 grant)]

Backgrond: This is a prospective multi-centric study undertaken at NIRT, Chennai and St. John's National Academy of health sciences, Bangalore.

Aims: (i) to determine the incidence and risk factors for dyslipidemia, abnormalities in glucose tolerance and body shape abnormalities in HIV-infected children between the ages of 2 and 10 years after 12 months after initiating anti-retroviral treatment (ART) and

(ii) to determine the role of genetic factors in the development of fat redistribution, insulin resistance and dyslipidemia in HIV-infected children after 12 months of ART in south India

Methods: HIV-infected children who are about to initiate ART are recruited. Details about their demographics, clinical & dietary history (Food security questionnaire, 24 hour dietary recall), physical examination, including anthropometric measurements are collected at baseline. They are followed every 3 months upto 12 months after initiation of ART. Blood investigations to measure lipid profile, peripheral insulin resistance, C-reactive protein, hematology, CD4 cell counts and viral load measurements are done at baseline, 6 and 12 months. The following single nucleotide polymorphisms are studied:

APOA5 (64G>C & -1131T>C), APOC3 (-482 C>T, -455 T>C & 3238 C>G), APOE (E2, E4 alleles), CETP (279 G>A, -629 C>A) and PLIN (6209T>C, 11482G>A, 13041A>G & 14995A>T)

The study was initiated in June 2011. As on 31st July 2012, 87 children have been recruited to the study. The sample size is 440 (220 at each site) and the study is ongoing.

HIV Vaccine Trial

Analysis of DNA prime-MVA boost Phase I HIV vaccine trial

(Principal Investigator: Dr.V.D. Ramanathan)

A randomized, double-blind, placebo controlled phase I trial was conducted in 32 HIV-uninfected healthy volunteers to assess the safety and immunogenicity of prime-boost vaccination regimens with either 2 doses of DNA (ADVAX) prime and 2 doses of recombinant MVA (TBC-M4) boost (Group A) or 3 doses of TBC-M4 alone (Group B). Both vaccine regimens were found to be generally safe and well tolerated. The breadth of anti-HIV binding antibodies and the titres of anti-HIV neutralizing antibodies were significantly higher ($p<0.05$) in Group B volunteers at 14 days post last vaccination. Neutralizing antibodies were detected mainly against Tier-1 subtype B and C viruses. HIV-specific IFN-γ ELISPOT responses were infrequent after ADVAX vaccinations, but were detected in all 12 Group A vaccinees after 1st and 2nd TBC-M4 injections. Six volunteers from Group B showed positive IFN-γ ELISPOT responses after 1st and 2nd TBC-M4 injections and 11/ 12 after the 3rd vaccination. The IFN-γ ELISPOT responses were directed mostly to Env and Gag proteins. The response rate, breadth and magnitude of IFN-γ ELISPOT responses did not differ significantly between the groups at the end of vaccination schedules. The responses persisted in 8/11, 8/12, 3/10 volunteers from Group A and 7/12, 4/10 and 4/11 volunteers from Group B at 3, 6 and 9 months post-last vaccination to TBC-M4 matched peptides, respectively. Enhancement of T-cell immune responses to DNA priming deserves further investigation using new technologies such as DNA administration by electroporation and /or molecular adjuvants.

Socio-behavioral studies

Studies in progress:

(i) HIV prevention through mobile phone technology among male sex workers in India (International collaborative studies)

(Principal Investigator: Dr. Beena E. Thomas, Source of Funding- Indo-US Joint Working group)

Background: This proposal is an outcome from a collaborative intervention study done in NIRT with the Harvard Medical School to address psychosocial needs of men having sex with men (MSM). Sex work is a matter of concern among this group, with elevated levels of sexual risk behaviour, inconsistent condom use and high prevalence of HIV and sexually transmitted infections (STIs). Our prior formative work revealed that the vast majority of sex workers in India have mobile phones and use these to network with "pimps" (individuals who manage male sex workers (MLSWs) and mediate client interactions), other sex workers, and to schedule sex work clients.

Aims: (i) to develop a HIV risk reduction counseling intervention for MLSWs in India using mobile phone technology,

(ii) to examine, in a pilot randomized controlled trial, the feasibility, acceptability and potential impact of the proposed intervention and

(iii) to assess if potential mediators of intervention differentially change in the intervention group and if these changes are associated with the primary outcome (reduced sexual risk taking)

The study has been initiated.

(ii) Health seeking behaviour and awareness of TB among migrants – Brick kiln workers - A study from Tiruvallur District, Tamil Nadu, India

(Principal Investigator: Mrs. Niruparani Charles, Source of Funding- Model DOTS Project)

Background: Migration has been reported as one of the main reasons for default. Brick kiln workers are one such group in Tiruvallur district who come here to work from adjoining areas between January to July each year. They are

hard to reach as they are confined in their brick kiln for long hours and these kilns are usually located on the outskirts. Furthermore, these workers are exposed to smoke and dust, have problems with regard to smoking and alcohol and little is known about their health care-seeking behavior and their awareness on TB.

Aims: (i) to understand the knowledge, attitude and perceptions on TB among brick kiln workers,

(ii) to identify the prevalence of chest symptomatics and health seeking behavior among brick kiln workers and

(iii) to find out the perceptions of the health providers in providing treatment and management of TB with special reference to brick kiln migrant workers

Methods: This is a cross sectional study which has been planned in 2 phases following a sequential approach. We have completed the first phase of the study which is,

- Enumeration of the recent list of brick kiln chambers in the Tiruvallur district
- Random selection of chambers and calculation of sample size (Sample size=3850)

Progress of the study: We have completed 40 chambers covering a population of 2800 brick kiln workers. We have also conducted seven in-depth interviews with Medical officers and two focus group discussions with health care providers. The study is ongoing.

(iii) An experimental study to enhance treatment adherence in TB patients who consume alcohol

(Principal Investigator: Dr. Mohanarani Suhadev, Source of Funding- Model DOTS Project)

Background: This study is an outcome of a previous pilot study conducted during 2009-2010 to explore suitable intervention measures for TB patients with Alcohol Use Disorders (AUD) in Chennai Corporation, Tamil Nadu. The pilot study revealed that 29% of the 490 TB patients consumed alcohol. The prevalence of AUD among them was 52%. The qualitative component of the study highlighted the need for an intervention among TB patients with AUD and

the feasibility and acceptability of an intervention. This Randomized Controlled Trial (RCT) experimental intervention study has been planned to enhance treatment adherence in TB patients who consume alcohol.

Objectives:

(i) to enhance treatment adherence of TB patients who consume alcohol by reducing the default rate through intervention strategies

(ii) to evaluate the impact of intervention strategies by comparing the treatment adherence of TB patients with disorders related to alcohol use in the experimental area with those TB patients with disorders related to alcohol use in the control area

The findings will provide the necessary information on enhanced treatment adherence due to alcohol intervention measures and help the Revised National ontrol Programme (RNTCP). This information could help develop a standard intervention model that could be feasible and acceptable in programmatic conditions to improve adherence among TB patients.

Progress of the study: We have screened 1212 TB patients and recruited 540 patients. The study is ongoing.

(iv) A pilot study to test the feasibility and impact of an intervention for mothers living with HIV/AIDS

(Principal Investigator: Dr. Beena E. Thomas)

Background

This was an outcome of a previously conducted Indo-US study in which a community-based approach to design an HIV/AIDS program for mothers living with HIV (MLH) was used.

The previous study had brought out key areas that challenge MLH which included barriers to accessing care, stigma from health providers, disclosure issues, legal issues, nutrition and coping with the illness. The present pilot experimental intervention study was initiated keeping in mind the felt needs of the MLH. The strategies adopted were based on the suggestions of the MLH.

Aim: To compare the Intervention Care group with the Standard Care group with respect to

- Physical health status
- Mental well being
- Social and family support
- Quality of life

Methods: This community-based, prospective, randomized, experimental two-group design randomly assigned 32 MLH to intervention and 32 MLH to a standard program. The intervention program designed through a community participatory approach covered topics on stigma, disclosure issues, adherence to ART, nutrition, legal issues, coping strategies and sharing personal experiences. There were five monthly sessions delivered over five months. Eighty one percent of the participants have completed the 3-month assessment and 72% of the participants completed the 6-month assessment.

Progress of the study: The study has been completed and data analysis is in progress. The study findings would help to plan a larger experimental study to assess the impact of the intervention model on the quality of life of MLH.

Laboratory studies

Clinical Pharmacology

Completed studies:

(i) Pharmacokinetics of first-line anti-TB drugs in children

(Principal Investigator: Dr. Geetha Ramachandran)

(Collaboration: Institute of Child Health, Chennai, Govt. Hospital of Thoracic Medicine, Chennai, Kilpauk Medical College & Hospital, Chennai and Govt. Rajaji Hospital, Madurai)

(Funding: ICMR Task force on Pediatric HIV)

Background: Current recommendations for pediatric dosages of anti-TB drugs are based on a small number of pharmacokinetic studies, few of which include younger children. In the RNTCP in India, ATT is given thrice-weekly and dosages of drugs are based on body weight. The importance of adequate dosing and therapeutic blood levels of anti-TB drugs has received attention in the light of recent reports suggesting that the currently recommended dosages of rifampicin (RMP), isoniazid (INH), PZA and ethambutol (EMB) are inadequate in children. During early life, children experience significant changes in the relative sizes of their body compartments and their ability to absorb, distribute, metabolize and excrete drugs. Genetic polymorphisms could also have a profound influence on INH and RMP drug levels. Not much is known about the impact of nutritional status on anti-TB drug pharmacokinetics.

Aims: (i) to evaluate the pharmacokinetics of RMP, INH and PZA in HIV uninfected children with TB who were receiving ATT according to the RNTCP guidelines in India and

(ii) to relate drug pharmacokinetic parameters with treatment outcomes

Methods: HIV seronegative children aged 1 to 12 years attending the RNTCP (TB treatment) centres at the Institute of Child Health, Chennai, Government Hospital of Thoracic Medicine, Chennai, and Government Rajaji Hospital, Madurai, meeting the study criteria were recruited. All the children were diagnosed of TB (pulmonary & extra pulmonary) using programme definition.

They were receiving ATT according to RNTCP guidelines from pediatric patient-wise boxes for a minimum of 2 weeks (six doses). The study commenced after obtaining approval from the Institutional Ethics Committees of all the study sites. Phenotypic INH acetylator status was determined. Nutritional status was assessed using z scores. During the intensive phase of ATT, a complete pharmacokinetic study was performed after directly observed administration of drugs. At two and six months, drug levels were checked 2 hours post-dosing. Plasma concentrations of RMP, INH and PZA were measured by High performance liquid chromatography (HPLC) and pharmacokinetic variables calculated. Multivariable regression analysis was done to explore factors impacting drug levels and treatment outcomes.

Results: A total of 84 children took part in the study. Table 5 shows the demographic and clinical characteristics of these children. The numbers of slow and rapid acetylators of INH were 57 and 27 respectively.

Impact of age: Peak concentration and exposure of all RMP, INH and PZA were significantly lower in children in the age range of 1 - 3 years compared to the other age groups ($p < 0.01$); however, drug pharmacokinetics were similar in the three older age groups. Ninety percent (76 of 84) of children had sub-therapeutic RMP peak concentration (< 8µg/ml) across all the age groups (all children under 3 years had RMP concentrations below 8µg/ml). In the case of INH and PZA, 10 / 84 (12%) and 31 / 84 (37%) respectively had sub-therapeutic peak concentrations (INH < 3µg/ml; PZA < 35µg/ml). There were a significantly higher number of children aged 1 – 3 years with sub-therapeutic concentrations than those aged 3.1 – 12 years (INH: 6 /10 vs. 10 / 74; $p = 0.003$ & PZA: 14 / 31 vs. 2 / 53; $p < 0.001$).

Impact of Nutritional Status: Peak concentration and exposure of RMP, INH and PZA were lower in children with stunting and underweight compared to normal children, the differences being statistically significant for RMP, INH and PZA for stunting and RMP and PZA for underweight ($p < 0.05$) (Fig. 2). No significant difference in peak concentration and exposure of RMP, INH and PZA was observed in children with wasting compared to normal children (Fig. 2).

Treatment Outcome: TB treatment outcomes were available in 70 children; 14 children had migrated and could not be followed up. Among the 70 children, there were 15 with unfavourable outcomes (death – 1; relapse – 1; failure - 13). Peak concentration, exposure, 2^{nd} and 6^{th} month concentrations of RMP and INH were significantly lower in children with unfavourable outcomes compared to those with favourable outcomes (Table 6). There was a delay in the time to attain peak concentration of RMP in the unfavourable responders compared to favourable responders; $p = 0.03$. Favourable outcome was achieved in 75% of cases when RMP, INH and PZA peak concentrations were within the therapeutic range. This dropped to 56% when all the drugs were below the therapeutic range; this difference however was not significant. A higher proportion of children > 3.1 years had a favourable outcome compared to those aged 1 – 3 years (84% vs. 54%; p=0.016). There was also a significantly higher proportion of slow acetylators of INH among children with favourable outcome (89% vs. 60%; $p = 0.005$).

Factors influencing drug levels: Multiple regression analysis was performed to test the influence of factors such as age, body mass index (BMI), serum albumin, INH acetylator status, stunting, underweight and wasting on peak concentration and exposure of RMP, INH and PZA. Results showed that age and WAZ (underweight) significantly influenced peak concentration and exposure of RMP, INH and PZA ($p < 0.05$). In addition, acetylator status had a significant influence on the pharmacokinetics of INH ($p < 0.05$).

Factors influencing treatment outcome: In univariate analysis, age, INH acetylator status, peak concentration, exposure, 2- and 6-month levels of RMP and INH were found to be significantly associated with treatment outcomes, $p < 0.05$. Multivariate logistic regression by stepwise method showed that only peak concentration of RMP (AOR 1.7; p=0.03) and 6 month INH concentration (AOR 2.2; p=0.005) significantly impacted treatment outcome.

Conclusions: This is the first study to report on the pharmacokinetics of RMP, INH and PZA in children with TB being treated with short-course, intermittent regimens, and also the first to relate drug levels with treatment outcomes. This

study has demonstrated that age (<3 years) and nutritional status (stunting and underweight) could influence drug pharmacokinetics, and that these could in turn influence treatment outcome. Regular monitoring of drug levels particularly in younger children and those with malnutrition could help in better patient management. Future recommendations for anti-TB treatment in children should consider age and nutritional status in order to achieve optimal treatment outcomes. The study findings of low anti-TB drug levels in children receiving standard short course chemotherapy (SCC) according to RNTCP guidelines in India and its impact on treatment outcomes, highlights the importance of considering children's dosage requirements separately.

Table 5: Demographic & clinical features of study participants (n = 84)

Details	**Mean ± SD**
Age (years)	7.1 ± 3.3
Males (n)	40
Body wt (kg)	18.8 ± 7.2
Nutritional status	
Height for age z score (HAZ)	- 1.2 ± 1.3
Weight for age z score (WAZ)	- 1.7 ± 1.0
Weight for Height z score (WHZ)	- 1.2 ± 1.1
Mid-arm circumference (cm)	
Head circumference (cm)	
Thrice weekly treatment dose mg/kg	
RMP	9.7 ± 2.2
INH	9.7 ± 2.2
PZA	32.0 ± 8.9
Duration of ATT (months)	0.8 ± 0.3
Regimen (n)	
Category I	48
Category II	3
Category III	33
Type of TB (n)	
Pulmonary	19
Extrapulmonary	63
Both	2
Rapid acetylators of INH (n)	27

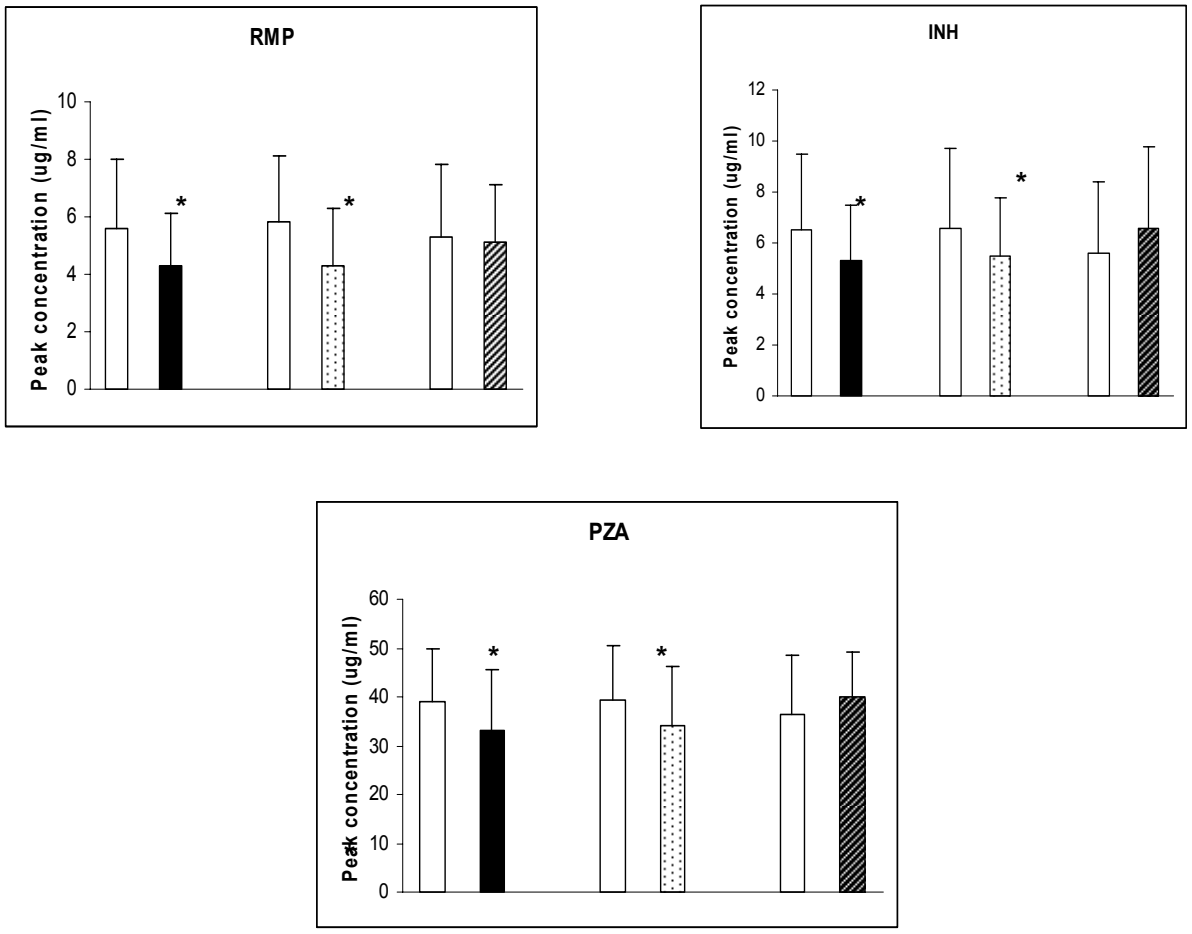
Table 6: Comparison of drug levels between children with favourable and unfavourable TB treatment outcome

	N	Unfavourable outcome	N	Favourable outcome	P Value
RMP					
C_{max}	15	3.8 ± 2.3	55	5.7 ± 2.2	0.01
$AUC_{(0-8)}$	15	17.5 ± 10.3	55	27.5 ± 11.8	0.01
T_{max}	15	3.6 ± 1.5	55	2.6 ± 1.1	0.03
2 month (2-hr)	14	2.9 ± 2.0	48	4.4 ± 2.3	0.02
6 month (2-hr)	14	1.0 ± 1.0	47	3.6 ± 2.4	0.001
INH					
C_{max}	15	4.7 ± 2.6	55	6.4 ± 2.7	0.03
$AUC_{(0-8)}$	15	17.3 ± 9.7	55	26.0 ± 11.9	0.01
T_{max}	15	2.8 ± 1.5	54	2.3 ± 0.8	NS
2 month (2-hr)	14	2.7 ± 2.1	48	6.4 ± 3.3	0.001
6 month (2-hr)	14	1.6 ± 1.4	47	4.9 ± 2.7	0.001
PZA					
C_{max}	15	32.8 ± 12.8	55	37.8 ± 11.3	NS
$AUC_{(0-8)}$	15	188.7 ± 70.0	55	215.5 ± 67.4	NS
T_{max}	15	3.2 ± 1.7	55	2.9 ± 1.4	NS

C_{max} – Peak concentration; Tmax - Time to attain peak concentration;

AUC – Exposure; NS – non-significant

Fig. 2: Stunted and underweight children have significantly lower peak levels of anti-TB drugs (Values are mean; vertical bars denote SD)



*** P < 0.05 vs. normal group**

- Normal; - Stunted; - Under weight; - Wasted

(ii) Determination of content of anti-TB drugs stored in different treatment centres in the RNTCP in Tamil Nadu: An observational study

(Principal Investigator: Dr. Geetha Ramachandran)

Funding: Model DOTS Project

Background: In India, the RNTCP provides decentralised treatment through a network of more than 400, 000 DOT providers, to provide TB treatment to the patients as near to their home as possible. Adverse storage conditions could affect drug content. EMB is known to become hygroscopic if not stored properly. In the case of Cs, a second-line anti-TB drug, gross deterioration under tropical climatic conditions has been reported from NIRT. It is important to ensure that patients receive good quality drugs, since poor quality of anti-TB drugs is one of the reasons for development of MDR-TB. Systematic quality control analysis of anti-TB drugs are required to improve cure rates and have a successful TB control programme.

Aim: To determine the content of certain anti-TB drugs available at different TB treatment centers of the RNTCP in the state of Tamil Nadu

Methods: This study was undertaken in eight districts in the state of Tamil Nadu, India. These districts were spread across the state with varying geographical terrain, accessibility and availability of DOTS plus drugs. In each district, drugs were collected at different settings such as, District TB centre, one TB Unit (TU), two designated microscopy centres (DMC) and DOT providers. A maximum of 10 each of the following drugs were collected from each setting:

(i) RMP(150mg & 450mg)
(ii) INH (300mg)
(iii) PZA (500mg & 750mg)
(iv) EMB (400mg & 600mg)
(v) Ethionamide (Eth) (250mg)
(vi) Levofloxacin (LFX) (500mg)
(vii) Cs (250mg)

At the time of drug collection, details such as manufacturing source, dates of manufacture & expiry, batch number and storage conditions were collected. For

analysis, drugs stored in the State drug stores (SDS) and drugs purchased and used in NIRT were also included in the study.

Drug estimations for their active ingredient were undertaken according to validated spectro-photometric methods after coding the tablets. The acceptable limits for content of the active ingredient applied to the individual drugs were taken as 90% - 110% (WHO report, 2011).

Results: Table 7 shows the mean drug content and % of drugs within the acceptable range in all the districts. The content of RMP 450mg, INH 300mg, PZA 750mg & 500mg, EMB 600mg & 400mg and Eth 250mg were within acceptable limits in all the districts. The drug content did not differ among districts, and that the geographical location of the districts did not influence the drug content. The number of tablets not within acceptable range was higher for RMP 150mg, Levofloxacin (LFX) 500mg and Cs 250mg.

Rifampicin 150mg: A total of 80 capsules from eight districts were analysed. Fig. 3 shows the distribution of individual RMP 150mg capsule content in each district. The mean drug content was 154mg (range: 102 – 277mg). Overall, 80% of capsules were within the acceptable range; the values were below the acceptable limits in five out of eight districts (70% in four districts & 80% in one district). About 83% of capsules from NIRT were within acceptable limits.

Cycloserine 250mg: A total of 70 Cs tablets from eight districts were analysed. Fig. 3 shows the distribution of individual Cs tablet content in each district. The mean drug content was 200mg (range: 108 - 245mg). Overall, only 21% of tablets from the districts were within acceptable limits; the values were below acceptable limits in all the districts. The percent of tablets falling within the acceptable limits ranged from 0 to 40%. About 40% and 68% of tablets from the SDS and NIRT respectively were within acceptable limits.

Levofloxacin 500mg: A total of 67 tablets from seven districts were analysed. Fig. 3 shows the distribution of individual LFX tablet content in each district. The mean drug content was 475mg (range: 365 - 515mg). Overall, 87% of tablets were within acceptable limits; the values were below acceptable limits in three

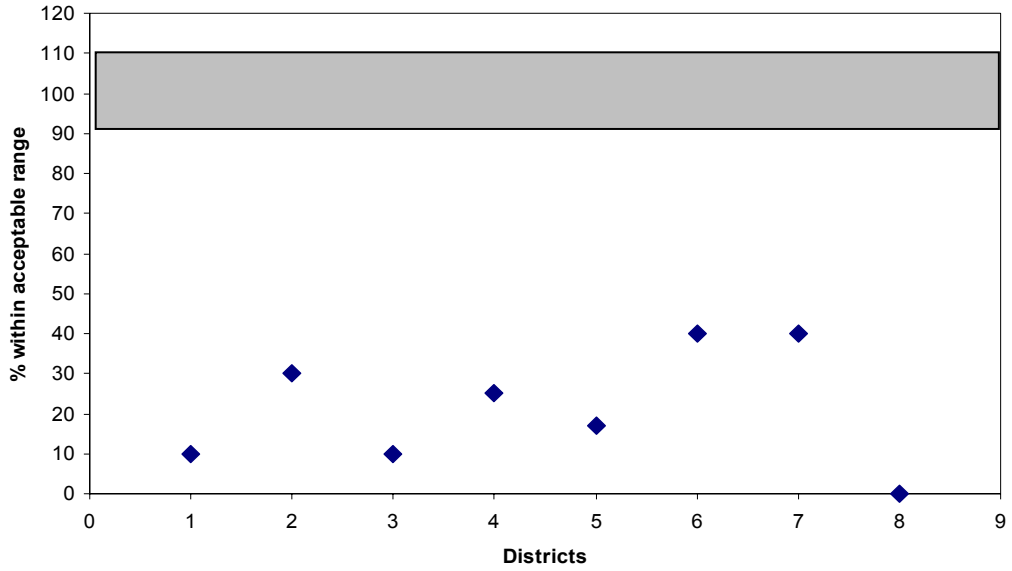
out of seven districts. About 77% of tablets from the SDS were within acceptable limits.

Conclusions: This is the first systematic study to report on the content of anti-TB drugs available in the RNTCP centres in the state of Tamil Nadu. Most of the drugs had their content within the acceptable range, which is quite encouraging. Low content of Cs observed in all the districts is a matter of concern. Although the RNTCP takes every effort to procure good quality drugs, varying storage conditions in different settings, particularly in DOT providers' homes (most of them lived in thatched huts) could have a bearing on the quality of drugs, particularly Cs. Periodic quality checks of anti-TB drugs available at the RNTCP centres are necessary to ensure that the programme continues to provide good quality anti-TB drugs to patients.

Table 7: Mean drug content & % adherence to stated content in 8 districts

Drug	No. of tablets	Mean (mg)	Min (mg)	Max (mg)	% drugs within acceptable range (90 – 110%)
Rifampicin 450mg	398	445	291	508	92
Rifampicin 150mg	80	154	102	277	80
Isoniazid 300mg	406	295	216	350	96
Ethambutol 600mg	346	601	406	842	97
Ethambutol 400mg	94	404	344	458	97
Pyrazinamide 750mg	406	722	137	857	94
Pyrazinamide 500mg	17	500	480	514	100
Cycloserine 250mg	70	200	108	245	21
Ethionamide 250mg	64	241	230	254	100
Levofloxacin 500mg	67	475	365	515	87

Fig. 3: Percent of cycloserine 250mg tablets within acceptable limits. The shaded area represents the acceptable range (90% - 110%)



Studies in progress:

(ii) Pharmacokinetics of anti-TB drugs in HIV-infected children with TB (Principal Investigator: Dr. Geetha Ramachandran)

Collaboration: Institute of Child Health, Chennai, Govt. Hospital of Thoracic Medicine, Chennai, Kilpauk Medical College & Hospital, Chennai and Govt. Rajaji Hospital, Madurai; Funding: ICMR Task force on Pediatric HIV)

Background: There is a paucity of pharmacokinetic data on existing anti-TB drugs given to children with HIV-TB co-infection, who also exhibit age-related differences in drug absorption, metabolism and clearance. HIV infection combined with intestinal dysfunction and malnutrition could cause impaired absorption of anti-TB drugs leading to reduction in blood levels of the drugs that can result in poor treatment response.

Aim: To study the influence of HIV infection on the pharmacokinetics of RMP, INH, PZA and EMB in children with HIV & TB

Methods: The study population includes two groups of children.

Group 1: TB

Group 2: HIV-TB

The required sample size is 80 in each group.

Children aged 1 to 12 years receiving treatment for TB from the RNTCP (TB treatment) centres at the Government Hospital of Thoracic Medicine, Chennai, Kilpauk Medical College & Hospital, Chennai, Institute of Child Health, Chennai and Government Rajaji Hospital, Madurai are recruited.

The INH acetylator status using saliva is determined. The nutritional status is assessed using z scores. The pharmacokinetic study is undertaken in the respective hospitals. On the day of the study, serial blood samples at pre-dosing and at 2, 4, 6 and 8 hours of blood (2ml) is collected followed by supervised drug administration. Wherever possible, RMP and INH concentrations at two and end of treatment (2 hour post-dosing) are determined. Plasma concentrations of RMP, INH and PZA are measured by HPLC and pharmacokinetic variables calculated. TB treatment outcomes are noted from the RNTCP card.

We have recruited 84 and 52 children respectively with TB and HIV-TB. Recruitment of HIV-infected children with TB is in progress.

(iii) Comparative pharmacokinetics of RMP during daily and intermittent dosing in HIV-TB patients

(Principal Investigator: Dr. A. K. Hemanth Kumar)

Collaboration: Govt. Hospital of Thoracic Medicine, Chennai

Background: This is a prospective pharmacokinetic study nested within a randomized controlled clinical trial in which HIV-infected patients with TB receive RMP along with other medications either daily or intermittently. It has been reported that intermittent RMP therapy in HIV-infected patients increases the risk of acquired RMP resistance among patients who fail. Sub-therapeutic plasma RMP could lead to unfavourable TB treatment outcome.

Aim: To study the pharmacokinetics of RMP in HIV-infected patients with TB who are receiving daily and intermittent anti-TB regimens

Methods: The study is conducted in a sub-set of 48 patients (24 each in the daily and intermittent dosing arms) who are getting recruited to the clinical trial. Eligible subjects are identified by the Clinicians involved in the trial. On the day of the study, blood samples are collected at 0, 1, 2, 6, 8, 12 and 24 hours after dosing. Plasma concentrations of RMP are estimated by HPLC according to a validated method. So far, 21 patients have been recruited to the study. The study is in progress.

(iv) Pharmacokinetics of rifabutin in HIV-infected TB patients

(Principal Investigator: Dr. Geetha Ramachandran)

(Collaboration: Govt. Hospital of Thoracic Medicine, Chennai)

Background: TB is the most common opportunistic infection and is an entry point for a significant proportion of HIV-infected patients who are eligible for ART. It is therefore a common scenario that HIV-infected patients eligible for ART also have concomitant TB and require treatment for both infections. Current recommendations are to treat patients with HIV-related TB with a regimen including a rifamycin for the full course of ATT. The rifamycin, rifabutin (RBT) is recommended for use in HIV-infected TB patients who receive second-line ART

regimens containing lopinavir / ritonavir. Concomitant use of RBT with PI-based antiretroviral therapy has been reported to lead to successful treatment outcomes. However, ritonavir (RTV), being a CYP3A4 inhibitor markedly increases serum concentrations and toxicity of RBT. It therefore becomes necessary to decrease the dose of RBT when co-administered with RTV. There have been conflicting reports regarding the dose of RBT during concomitant RTV administration. In India, the current practice is to reduce the dose of RBT by 50% in patients receiving concomitant RTV. There is not much pharmacokinetic data available to support this reduction.

Aim: To study the pharmacokinetics of RBT at the dose of 150mg thrice weekly during concomitant RTV administration in HIV-infected TB patients

Methods: This study is conducted at the Government Hospital of Thoracic Medicine, Chennai. Patients meeting the following inclusion criteria are recruited to the study:

Inclusion criteria

1. HIV-infected TB patients with a diagnosis of PTB based on sputum smear positive for *M. tuberculosis*
2. Receiving standard ATT with RBT-containing regimens regularly for minimum 2 weeks
3. Should have failed virologically to the first-line ART regimens and be receiving second-line regimens containing LPV/RTV regularly for minimum 2 weeks before enrolment
4. Should be adults $\geq$ 20 years
5. Body weight $\geq$ 30 kg
6. Willing to accept hospitalization
7. Willing to give informed written consent

On the day of the pharmacokinetic study, serial blood samples are collected at pre-dosing and at 1, 2, 4, 6, 8, 12 and 24 hours after drug administration. Estimation of plasma RBT, LPV and RTV are undertaken by HPLC according to validated methods. So far, 12 patients have been studied. The study is in progress.

(v) Monitoring plasma RMP and INH in HIV-infected patients with TB

(Principal Investigator: Dr. A. K. Hemanth Kumar)

Background: Treatment of TB in HIV-infected patients is an important issue in terms of both duration as well as frequency of administration. This becomes more complicated due to malabsorption of anti-TB drugs, especially RMP during HIV infection, resulting in subsequent treatment failure. This raises an important question of whether HIV-infected patients require therapeutic drug monitoring and, if required, increase the dosages of anti-TB drugs, in order to overcome the effect of malabsorption.

Aim: To determine plasma RMP and INH concentrations in HIV-infected patients with TB and correlate with TB treatment outcome

Methods: This study is done in all patients who are participating in an ongoing randomized controlled clinical trial at NIRT. Blood samples at 2-hour post dosing are collected at months 1 and 5 of ATT. Plasma RMP and INH estimations are undertaken by HPLC in a blinded manner. Patients are followed up according to the trial protocol. So far, plasma samples at both time points have been collected from 41 patients. The study is in progress.

(vi) Development and validation of methods for estimation of anti-TB drugs by Liquid Chromatography Mass Spectrometry

(Principal Investigator: Dr. A. K. Hemanth Kumar)

Accurate and sensitive methods to estimate anti-TB drugs in plasma are necessary for the conduct of pharmacokinetic studies in adult and pediatric groups of patients. Methods by Liquid Chromatography Mass Spectrometry (LCMS) have an advantage of being highly sensitive requiring a low plasma volume for estimation. This would be useful, especially in young children from whom it is difficult to collect blood at multiple time points. At present, we run most of the drug estimations by HPLC. We are in the process of standardizing methods for estimation of RMP, INH, PZA and EMB by LCMS.

HIV studies

Studies in progress

(i) Predictors and immunologic characterization of TB-associated IRIS in a prospective clinical trial cohort in Chennai, India

(Principal Investigator: Dr. Soumya Swaminathan; Funding Agency: Intramural to India Grant (NIAID), National Institute of Health)

IRIS or paradoxical worsening of infection, following the initiation of ART is particularly common in AIDS patients with co-existent TB. In our efforts to predict this phenomenon, a prospective cohort study nested within a parent TB-treatment trial has been undertaken by NIRT in collaboration with the NIH. In this study, clinical predictors and risk factors are evaluated and compared between patients who develop TB-IRIS and those who do not, based on baseline characteristics. Markers of T-cell activation (e.g., PD-1, CD69, intracellular Ki67, co-expression of HLA-DR and CD38) are being evaluated for predicting TB-IRIS by comparing values at baseline and during TB-IRIS episodes. The effector response of CD4 T-cells to TB is studied by longitudinal follow-up of T-cell stimulation assays and a panel of serum cytokines (Th1/Th2/Th17) is measured. The study was started in November 2009. As on 01.03.12, 57 patients were enrolled, out of which 19 experienced IRIS. The median time to ART after ATT was 22 days (14-34 IQR). The median time to IRIS occurrence was 9 days (7-15 IQR) and to resolve was 11 days (8-15 IQR). Low CD4 cell counts and high viral load were predictive of this syndrome. Analysis of laboratory markers in patients with and without IRIS is in progress.

(ii) Frequency of drug resistance mutations among HIV-infected adults failing first line antiretroviral therapy in southern India

(Principal Investigator: Dr. Luke Elizabeth Hanna)

The current standard first-line treatment for HIV in India consists of two nucleoside reverse transcriptase inhibitors (NRTIs), zidovudine or stavudine plus lamivudine, and one nonnucleoside reverse transcriptase inhibitor (NNRTI), nevirapine (NVP) or efavirenz (EFV). Regimens with protease inhibitors (PIs) are available as second-line treatment options upon failure of the first-line ART under

the national program. Surveillance for emergence of resistance in HIV against the drugs recommended by the National Programme has been undertaken by the WHO HIV Resistance Monitoring Programme (HIVResNet) for HIV Drug Resistance Prevention, Surveillance and Monitoring Programme. The information obtained will help us to review the efficacy of the current treatment regimens and to plan or modify the composition of ART in order to derive maximum benefit from this intervention.

The present study aimed at determining the prevalence of drug resistant mutations in 50 HIV-1-infected individuals from southern India who failed standard first line antiretroviral therapy. The observed frequency of mutations conferring resistance to lamivudine was 82%, stavudine 60%, zidovudine 56%, EFV 66%, NVP 94%, didanosine and abacavir 48%, delavirdine 68%, emtricitabine 82%, zalcitabine 70% and tenofovir 8%, indicating a high frequency of NRTI and NNRTI mutations among this group of patients.

We also sequenced the protease and integrase genes to look for the presence of naturally occurring resistance mutations to PIs and integrase inhibitors. None of these samples had major resistant mutations to PIs as well as integrase inhibitors. However, two samples showed the presence of minor mutations (L74M and E138D) in the integrase gene.

(iii) Molecular characterization of HIV-1 subtype C isolates circulating in India

(Principal Investigator: Dr. Luke Elizabeth Hanna)

The HIV-1 epidemic in India is predominated by the subtype C virus, which is not only distinct from other HIV-1 subtypes, but also from subtype C viruses present in other parts of the world. It is therefore imperative to understand the unique biological and molecular properties of these viruses. The present study aimed to characterize some of the unique features of HIV-1C isolates circulating in our population.

Molecular determinants of co receptor usage are known to be present on the HIV envelope. Changes in cellular tropism by HIV-1 *in vivo* seems to be a key event in disease pathogenesis, and broadening of the co-receptor usage profile of HIV-

1 may be associated with accelerated CD4 T-cell loss, disease progression to AIDS. Published research suggests that HIV-1 subtype C isolates in different regions of the world isolated from various cohorts during different disease stages utilize CCR5 almost exclusively and show minimal conversion to CXCR4 tropism. Although the percentage of CXCR4 usage among HIV-1 subtype C strains is not as high as subtypes B and D (50–90%), there are indications that the frequency of subtype C CXCR4-utilizing viruses may be increasing with time. The present study is an attempt to determine the frequency of CCR5, CXCR4 and dual tropic phenotypes in HIV-1 isolates obtained from HIV-1 infected subjects in various stages of disease. Forty clinical isolates have been co-cultured and typed for co-receptor usage by means of a phenotypic assay using U87. CD4 cell lines expressing either CXCR4 or CCR5. Majority of the isolates used CCR5 as the co-receptor even during late stage disease. None of the isolates tested thus far were found to use CXCR4 exclusively. However, a few isolates were dual tropic and could use both co receptors. We also sequenced full length envelope genes of the viruses with a view to identifying molecular determinants for expanded coreceptor usage in HIV-1 subtype C isolates using more than one co-receptor.

HIV-1 Rev is an accessory protein which helps in the export of unspliced and partially spliced mRNA from the nucleus to the cytoplasm. A unique feature of HIV-1 subtype C is that it has rev genes of variable length, as a result of heterogeneity in the C-terminus. One of our objectives is to understand the functional significance of the C-terminus heterogeneity of HIV-1 subtype C Rev. Both exons of the rev genes of five different HIV-1 subtype C strains coding for Rev proteins ranging in size from 100 amino acids to 126 amino acids were cloned into a vector. A subtype B rev coding for a 116 amino acid long Rev protein was used as the control. The six constructs were assayed for rev activity by co-transfecting them into 293T cells along with a gagpol-RRE construct, first with a subtype B RRE belonging to NL4-3 (p-gagpol-RRE-B) and then with a subtype C RRE belonging to Indie-C1 (p-gagpol-RRE-C), and measuring gag production by measuring p24 antigen in the culture supernatant. We found that longer Revs appeared to function more efficiently than shorter ones, particularly

at low concentrations such as is seen *in vivo* in early stages of infection. We also found that subtype C RRE was a weaker activator of Rev activity than subtype B RRE, in spite of high levels of similarity between the two RRE sequences.

HIV-1 LTR encodes regulatory elements that determine the infectivity and replicative fitness of the virus. Molecular characterization of the LTR of HIV-1C isolates in our population is also being undertaken. The study is ongoing.

Department of Bacteriology

Completed study

(i) Viability and retrieval of *M. tuberculosis* from Kirchner's liquid medium using bovine serum albumin

(Principal Investigator: Dr. Gomathi Sekar)

Background: Kirchner's liquid medium (KLM), an enriched medium is used to isolate *Mycobacterium tuberculosis* (MTB) from extra pulmonary specimens. Fetal calf serum (FCS) is the commonly used enrichment source that enhances the growth of mycobacteria.

Aim: To substitute bovine serum albumin (BSA) for FCS in KLM and to compare the growth characteristics of colonies formed in Kirchner's containing FCS (KLM-FCS) and Kirchner's containing BSA (KLM-BSA)

Method: Standard suspensions (4mg/ml) from seven confirmed clinical isolates of MTB were prepared and serial ten fold dilutions were made. One hundred microliters from each dilution was used for inoculating a set of media containing - one LJ slope, one KLM with FCS, and one with BSA. Weekly readings for LJ medium were taken up to eight weeks and for liquid media up to six weeks following which they were decontaminated using modified Petroff's method and inoculated on fresh LJ medium and read up to eight weeks.

Results: All seven cultures showed detectable growth in both forms of KLM. Comparison of growth characteristics of both media revealed that MTB growth in KLM-BSA exhibited more typical morphology, were more confluent, quicker to form and supported growth up to the 6^{th} dilution. KLM-BSA also was found to be free of contamination.

Conclusion: Bovine serum albumin appears to be an effective enrichment alternative to FCS in the preparation of Kirchner's liquid medium as it yields rapid growth of MTB with clear morphology with less or little contamination. Factors

such as cost effectiveness, commercial availability, ease of storage makes BSA a more ideal enrichment source.

Studies in progress:

(i) Establishment of national database on TB

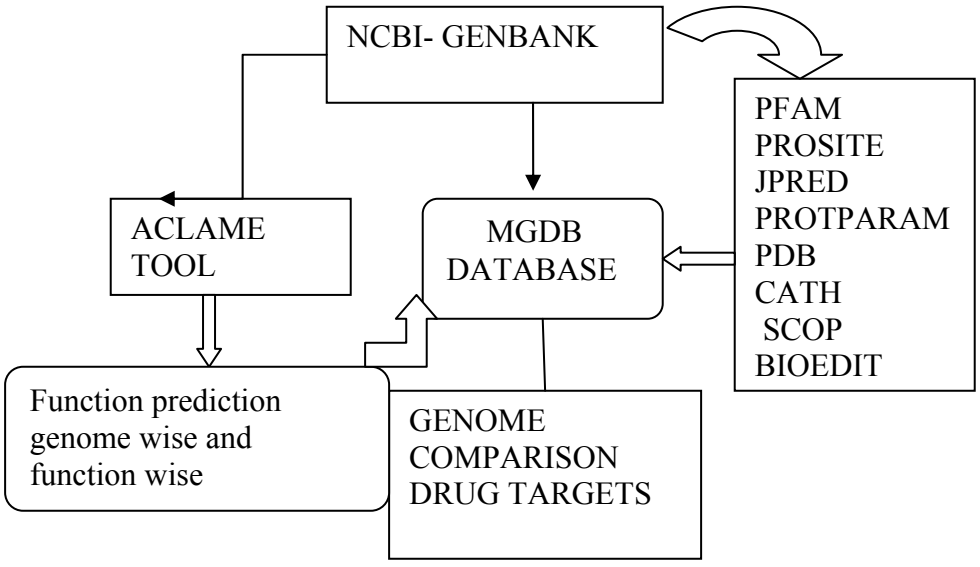
(Principapl Investigator: Dr. Vanaja Kumar)

Background: Mycobacteriophage genome database (MGDB) an exclusive repository of the 70 completely sequenced mycobacteriophages with annotated information has been developed. The MGDB (Version No.1.0) comprises of 8318 genes from 70 mycobacteriophages classified into 72 families based on ACLAME database. Manual curation was aided by information available from public databases which was enriched further by analysis. Its web interface allows browsing as well as querying the classification. BLAST tool has been incorporated into the database.

Aim: To collect data on mycobacteriophage genomes/proteomes and classifying the mycobacteriophage proteins in a rational way using MGDB

Methods: A schematic representation of the methodology adopted in this study is shown in Fig 4.

Fig. 4: Data flow diagram for mycobacteriophage genome database



Results: Phage classification using Phaemerator for mycobacteriophages has already been done and classified as clusters from A-O. This information has been integrated into the MGDB. Seventy mycobacteriophages containing 8120 genes (except tRNA) were classified into 7815 phamilies and added to our DB of which 37 mycobacteriophages have been subjected to CODON W, BIOEDIT and RSCU for studying codon bias. Such analysis will enable to decipher the factors contributing to codon bias. Digitalization of published articles from 1958-2009 (986 published) has been completed.

(ii) Characterization of anti-mycobacterial compound from less explored ecosystems towards novel drug discovery for TB

(Principal Investigator: Dr. Vanaja Kumar)

Background: Emergence of MDR forms of TB has triggered the search for novel drugs from less explored ecosystems. Crude extracts from marine actinomycete strain ANS2 was isolated from Andaman and Nicobar marine sediment. The extract was tested and found to have anti-bacterial activity. Isolation and characterization of active anti-TB compounds from the crude extract, is being carried out as a collaborative study between Periyar University (Salem), IIT and NIRT.

Aim: To isolate and characterize novel anti-TB molecules from ANS2 crude extracts that has shown anti-bacterial activity

Methodology: The crude extract was subjected to LRP assay. It was purified by thin layer chromatography (TLC) and the fractions obtained were again tested by LRP assay. The structure elucidation of all active compounds is being performed by Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared Spectroscopy (FTIR).

Results: The crude extract showed potential anti-TB activity against standard and clinical (drug sensitive, MDR and XDR) strains of *M. tuberculosis.* The physicochemical properties of the isolated active molecule showed it to be a yellow color substance with an rf value of 0.75. It is soluble in methanol and chloroform but insoluble in n-hexane. The FTIR using KBr showed a wavelength of Vmax (cm-1) 2923, 1732 values. The NMR results were obtained from SAIF

(IIT-Chennai). The novelty of the strain ANS2 is being explored based on 16s rRNA analysis and the structure elucidation of purified pigment is in progress.

(iii) TB drug resistance surveillance in the state of Tamil Nadu, India

(Principal Investigator: Dr. Vanaja Kumar)

Surveillance of TB drug resistance in the state of Tamil Nadu was initiated in June 2011 to determine the proportion of drug resistance in new and previously treated cases of PTB. This is used as a level of drug resistance as a performance indicator for the RNTCP. Based on cluster sampling, the sample size for new sputum smear positive cases (NSP) and re-treatment (RT) cases were determined to be 1680 and 992 respectively. Applying the WHO recommended Population Proportion to Size (PPS) method, 70 DMCs were randomly selected from a total of 700 for enrolling patients in the study. The sputum samples were transported from DMCs to the central laboratory without Cetyl Pyridinium Chloride as per the standard operating procedure developed by NIRT. Culture and susceptibility test for the isolates are performed as per the National DRS protocol developed by the Central TB Division. The economic variant of proportion sensitivity test is performed for susceptibility testing for two first line drugs, namely, INH and RMP and for two second line drugs, namely, OFX and kanamycin, for the simultaneous detection of MDR as well as XDR-TB. Currently, a total of 2412 sputum samples were received for the study of which 62% were from NSP cases and the remaining 38% from RT cases. Rates of drug resistance will be calculated after the completion of the study.

RNTCP activities in National Reference Laboratory, NIRT, Chennai (2011-12)

(Contact Person: Dr. Vanaja Kumar / Dr. N. Selvakumar)

NIRT, Chennai is functioning as a National Reference Laboratory for RNTCP. As part of the activity, 49 laboratory personnel were trained for culture and drug susceptibility testing (DST) from 25 different national and international institutes.

Accreditation of three laboratories have been completed (two by liquid based DST and one by solid culture based DST). Fifteen laboratories including Intemediate reference laboratory and private laboratories are under the process

of accreditation. Pre-accreditation assessment of seven laboratories has been completed. Six of the previously accredited laboratories have been reaccredited based on proficiency testing. A total of 362 follow up cultures have been received from different states of India and processed for second line DST. On-site evaluation of five states have been completed as part of EQA of sputum microscopy using 700 panel slides, and 140 laboratory personnel have been assessed. Drug Resistance Surveillance (DRS) study for Tamil Nadu was initiated in June 2011. Comprehensive training on case recruitment, sputum transportation etc. was given to 398 medical and paramedical personnel belonging to 68 designated microscopy centres in 31 districts. Utility of a simple method for packing and transportation of sputum samples (3P: pack per patient) has been demonstrated by the DRS study. A user friendly electronic system (e-MANDRS) was developed as a recording and reporting system for DRS survey.

Establishment of Line Probe Assay facility for rapid detection of MDR TB: World Health Organization recommends the use of Line Probe Assays for the rapid detection of rifampicin resistance directly from sputum samples. Performance of the assay requires an exclusive facility comprising of three clean rooms one each for the preparation of PCR mix, amplification and hybridization. Establishment of such a facility in the Department of Bacteriology, NIRT was coordinated by NIRT, WHO – SEARO Delhi and FIND India with financial assistance from PATH.

Department of Immunology

Completed studies:

(i) Use of alternative markers other than IFN-γ

(Principal Investigator: Dr. Alamelu Raja)

Background: The suboptimal sensitivity of Interferon (IFN)-γ-based *in-vitro* assays, especially in immunocompromised individuals, emphasizes the need for alternative markers for diagnosing TB.

Aim: To evaluate whether *in vitro* response to *M. tuberculosis* RD1 peptides selected by computational analysis, measured by IFN-γ, interferon-inducible protein (IP) - 10, monocyte chemotactic protein (MCP) - 2 and interleukin (IL) - 2 can be useful biomarkers associated with active TB, with and without HIV infection

Methods: One hundred and twenty nine individuals were prospectively enrolled, 41 with active pulmonary TB and 88 without TB. The latter group comprised of healthy household contacts (HHC) and community controls. Sixty-six HIV-infected individuals were prospectively enrolled, 28 with active PTB and 38 without TB. A whole blood assay based on RD1 selected peptides was performed. Soluble factors were evaluated by ELISA in plasma harvested on day 1 post-culture. Enrolled individuals were also tested by QuantiFERON TB-Gold In tube (QFT - IT) and tuberculin skin test (TST).

Results: IFN-γ response to RD1 selected peptides was significantly higher in active TB patients than in HHC and community controls (Fig. 5). IP-10 and MCP-2 response did not differ between active TB patients and HHC, although it was higher in these groups compared to community controls; conversely IL – 2 response did not differ among the three groups. When IFN-γ response to RD1 selected peptides was scored based on receiver-operator-characteristic analysis, active TB was predicted with 68% sensitivity and 86% specificity. While the sensitivity of QFT - IT and TST for active TB were 90% and 68%, the specificities were 58% and 59%, respectively. In HIV TB, by detecting IP-10, the sensitivities

of the RD1 selected peptides and QFT - antigen (75% and 86% respectively) for active TB were higher compared to the same assays based on IFN-γ (43% and 61% respectively). This was not influenced by the ability to respond to the mitogen (Fig. 6). By detecting IP-10, the specificity of the RD1 selected peptides and QFT-antigen (58% and 13% respectively) for active TB was lower than what was reported for the same assays using IFN-γ-detection (79% and 68% respectively).

Conclusions: IFN-γ (but not IP-10, MCP-2 and IL-2) response to RD1 selected peptides is associated with active TB with a higher specificity than QFT-IT and TST. HIV infection does not impair RD1-specific response detected by IP-10, while it significantly decreases IFN-γ mediated responses.

Fig. 5: IFN–γ production in response to RD1 selected peptides is associated with active TB

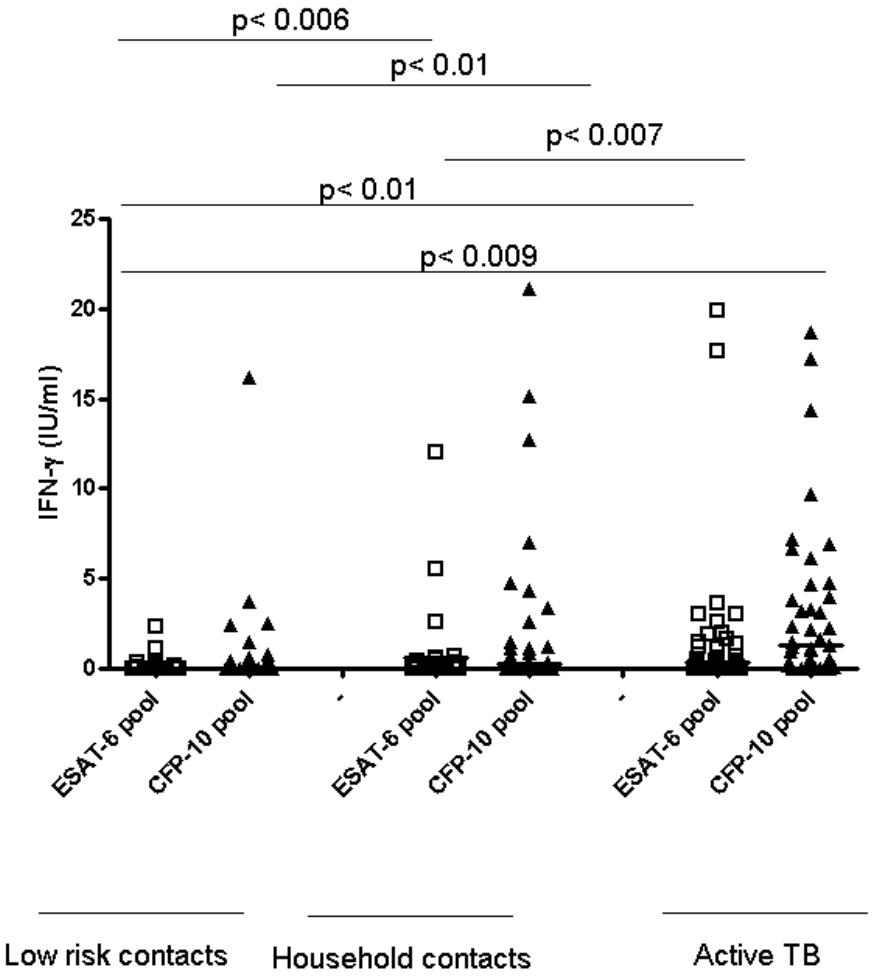
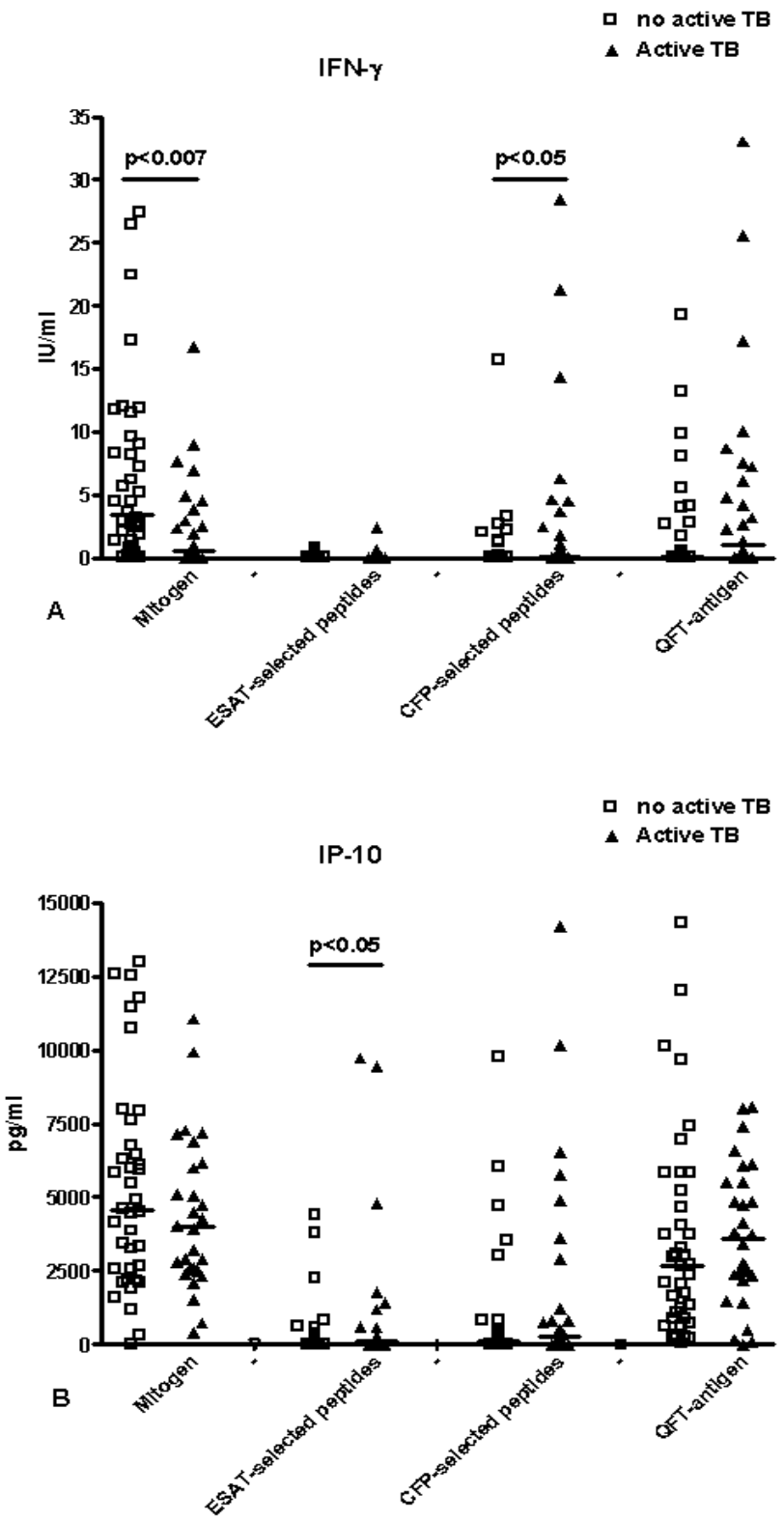


Fig. 6: IP-10 production in response to RD1 selected peptides and QFT-antigen in HIV+



(ii) Cytotoxic cell response in *M. tuberculosis* infection

(Principal Investigator: Dr. Alamelu Raja)

Background: Cytotoxic T lymphocytes are required for protective immunity against intracellular pathogens such as *M. tuberculosis*. These pathogens are known to escape from phagocytic vacuoles into the cytoplasm of infected host cells. Cytotoxic T-cells are needed to release bacteria from safe havens inside ineffective macrophages so that they can be phagocytosed by fresh, fully activated monocytes or macrophages. Earlier we have studied the cytotoxic cell response, epitopes involved in cytotoxicity and mechanisms of cytotoxicity.

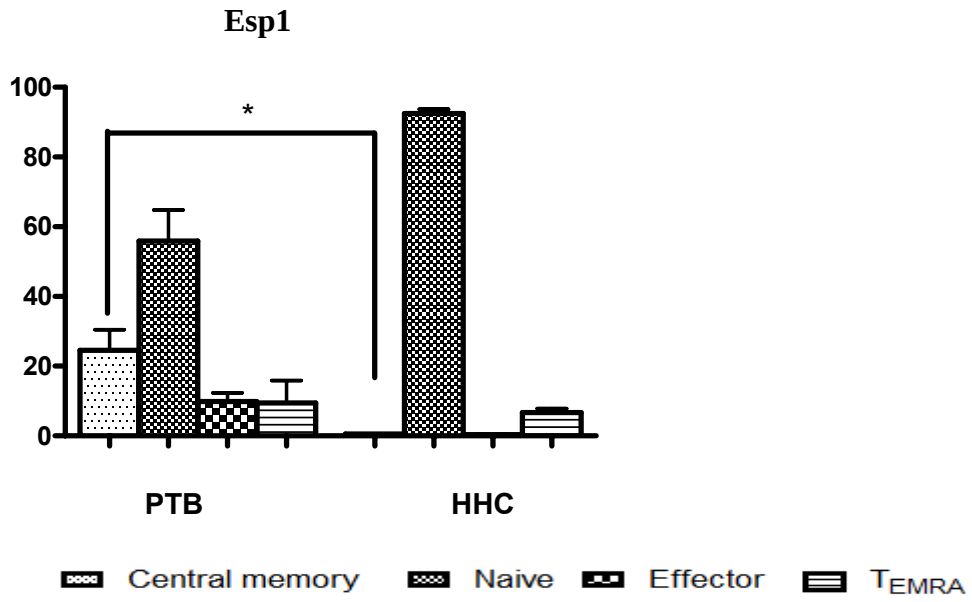
Aim: To analyse the memory cytolytic response to the selected epitopes

Methods: Memory cytolytic responses to selected ESAT-6 ($Esp1_{1-20}$, $Esp6_{51-70}$) and CFP-10 ($Cfp6_{51-70}$, $Cfp8_{71-90}$) peptides were studied in TB patients (n=15) and their HHC (n=15). These peptides were selected on the basis of IFN-γ/IL-4 ratio and bioinformatic prediction in our previous studies. The memory subsets and the cytolytic molecules secreted by them were studied by flow cytometry.

Results: Of the four memory subsets, central memory subset was enhanced by Esp1 (Fig. 7) and effector memory, by Cfp6 in PTB (Fig. 8). The peptide Esp6 caused an increase in T_{EMRA} subset in HHC. Cytolytic (Perforin and granulysin) central memory subset positive CD8 and CD4 T-cells were induced by Cfp8 in PTB. Cytolytic T_{EMRA} subset was increased by Esp1, Esp6 and Cfp8.

Conclusion: Further studies on these peptides may delineate the role of memory cell responses to these specific *M. tuberculosis* antigens which may help in designing an effective vaccine for TB.

Fig. 7: Memory subset responses to ESAT-6 by CD4+ cells



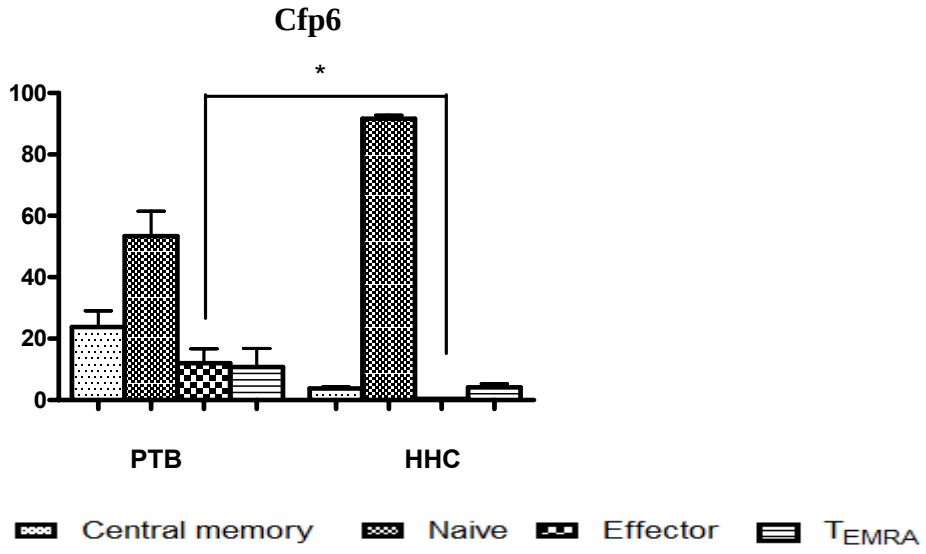
Each bar represents the mean of 15 subjects for each memory population.

PTB – Pulmonary tuberculosis patients

HHC – Healthy household contacts

T_{EMRA} – Terminally differentiated effector cells

Fig. 8: Memory subpopulation responses to CFP-10 by CD4+ cells



Each bar represents the mean of 15 subjects for each memory population.

PTB – Pulmonary tuberculosis patients

HHC – Healthy household contacts

T_{EMRA} – Terminally differentiated effector cells

(iii) Innate immunity in HIV infection

(Principal Investigator: Dr. Alamelu Raja)

Background: The Natural Killer (NK) T-cells express an invariant T-cell receptor and has been demonstrated to play an important regulatory role in a variety of immune responses. Although defects in the invariant NK T-cell population have been observed in patients with cancer and autoimmune diseases, little is known regarding its protective role in human infectious disease. The present study investigated the frequency and function of NK T-cells in HIV-infected individuals in addition to the receptor expression pattern.

Aims: To investigate the frequency and function of NK T-cells in HIV-infected individuals in addition to the receptor expression pattern

Materials and Methods: The study included 15 individuals each of normal healthy subjects, PTB patients, HIV-infected individuals and patients with HIV and TB co-infection. The frequency of NK T-cells and the expression of phenotype, cytotoxic and chemokine receptors were studied by flow cytometry.

Results: The number of invariant NK T-cells was significantly depleted in HIV and HIV-TB patients, which normalized in HIV upon IL-15 + IL-12 stimulation (Fig. 9). The constitutively expressed NK cytotoxicity receptor, NKp46 was elevated in HIV and HIV-TB individuals, which might be the host's response to HIV replication (Table 8). The distinct expression patterns of chemokine and adhesion receptors suggest that NK T-cell subsets might traffic to different tissues and different microenvironment. High expression of chemokine receptor, CCR5 by most NK T-cells suggests that these cells might be more favorable targets of HIV infection (Table - 8).

Conclusion: IL-15 and IL-12 combination have the ability to normalize the selective depletion of invariant NK T-cells in HIV-infected individuals, but of limited value when co-infected with TB.

Fig. 9: Enumeration of invariant NKT-cells and effect of cytokine stimulation

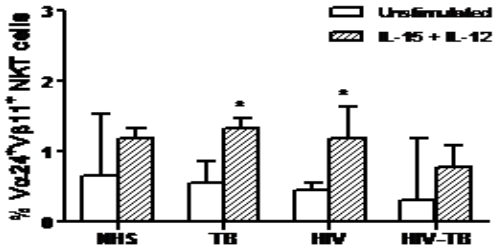


Table 8: Expression pattern of cytotoxicity and chemokine receptors on iNKT cells

Receptors	Stimulant	Median percentage of cells (IQR)			
		NHS	TB	HIV	HIV-TB
NKp30	Unstimulated	6.8 (5.9-19.6)	7.5 (6.4-36.7)	9.0 (8.6-22.5)	9.1 (1.8-15.5)
	IL-15 + IL-12	5.2 (4.0-11.4)	6.7 (1.9-13.4)	17.0 (8.2-27.9)	**20.7 (12.6-38.9) #**
NKp44	Unstimulated	9.5 (8.0-13.3)	17.1 (3.9-20.5)	9.6 (5.9-15.0)	16.0 (7.9-17.3)
	IL-15 + IL-12	16.0 (7.9-25.0)	8.9 (2.3-29.6)	12.2 (10.4-18.6)	**27.4 (9.0-36.1) #**
NKp46	Unstimulated	8.6 (2.5-12.1)	5.7 (3.5-21.2)	**19.2 (7.1-19.8) ***	**28.8 (4.5-42.7) ***
	IL-15 + IL-12	6.5 (3.7-18.9)	5.4 (3.0-33.8)	18.6 (3.7-29.6)	30.5 (16.0-50.5)
CXCR4	Unstimulated	74.4 (59.6-90.6)	61.4 (61.3-93.2)	61.3 (2.2-74.3)	52.7 (30.4-81.8)
	IL-15 + IL-12	55.5 (40.5-73.2)	57.5 (46.5-76.2)	51.8 (30.6-77.8)	40.8 (26.3-64.1)
CCR5	Unstimulated	23.3 (13.0-43.9)	29.7 (17.3-49.1)	**45.5 (20.6-55.6) ***	**46.5 (29.4-54.0) ***
	IL-15 + IL-12	17.3 (13.3-29.9)	**46.0 (27.8-64.0) #**	55.3 (24.5-78.3)	45.7 (24.3-61.6)
CCR7	Unstimulated	15.5 (8.9-53.3)	14.6 (5.6-22.7)	**7.4 (3.9-21.7) ***	**4.8 (2.8-22.6) ***
	IL-15 + IL-12	8.6 (7.0-45.3)	7.2 (4.4-15.7)	7.7 (6.0-22.9)	8.2 (7.3-19.2)

Data are presented as median (Inter Quartile Range) of 15 subjects. Statistical comparisons between NHS and other groups were performed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test and the significance is denoted by *. Statistical analysis between basal and IL-15 + IL-12 stimulation was carried out using Mann-Whitney U test and the significance is showed as #. A *p* value of <0.05 was considered statistically significant.

(vi) RANTES (CCL5) gene polymorphisms in PTB

(Principal Investigator: Dr.P. Selvaraj)

Background: Regulated on activation, normal T-cells expressed and secreted (RANTES/CCL5) is a chemokine which plays an important role in the formation of granuloma during infection with *M. tuberculosis*. Production of CCL5 is regulated by the polymorphic gene variants of CCL5 gene.

Aim: To find out whether polymorphisms in the promoter and intron region of CCL5 gene are associated with susceptibility or resistance to PTB in south Indian population

Methods: Polymorphisms in the promoter (-403 G/A- and -28C/G) and intron (In1.1T/C) regions of CCL5 gene were studied in 212 PTB patients and 213 healthy controls (HCs), using polymerase chain reaction based restriction fragment length polymorphism (RFLP) methods.

Results: Allele and genotype frequencies were not different between PTB patients and HCs. However, a significantly decreased frequencies of the haplotype A-C-C [P = 0.037; Odds ratio (OR): 0.57; 95% confidence interval (CI): 0.34–0.97] and the diplotype G/A-T/C (P = 0.017; OR: 0.46; 95% CI: 0.24–0.88) were observed among PTB patients when compared to HCs.

Conclusion: The results suggested that CCL5 gene polymorphisms examined in this study were not associated with PTB. However, the CCL5 halpotype A-C-C and the diplotype G/A-T/C may be associated with resistance to PTB.

Implications: The findings may be useful to understand genetic susceptibility and resistance to PTB.

(vii) Stromal cell derived factor-1 (SDF-1/CXCL12) gene polymorphisms in PTB

(Principal Investigator: Dr.P. Selvaraj)

Background: The chemokine, Stromal cell-derived factor-1 (SDF-1/CXCL12) regulates the trafficking of various types of leucocytes to areas of injury and infection. Polymorphisms in the CXCL12 gene influence CXCL12 levels and might be associated with the outcome of infection.

Aim: To find out whether polymorphisms in the intron and 3'untranslated region (UTR) of SDF-1/CXCL12 gene are associated with susceptibility or resistance to PTB

Methods: Genotyping of In2 +5887 (rs2839693) In2 +6201(rs266085) 3' UTR +12197 (rs1801157) 3' UTR +14478 (rs1065297) polymorphisms were investigated among 184 PTB patients and 187 healthy controls of south India using polymerase chain reaction (PCR) based RFLP methods.

Results: An increased frequency of G/A genotype of In2 +5887 [P = 0.034; OR 1.66; 95% CI 1.04–2.66] was observed among PTB patients than HCs. Moreover, significantly increased frequencies of G / A genotype (P = 0.013 $P_{corrected}$ = 0.039; OR 2.41) of In2 +5887 and G/G genotype (P = 0.005, $P_{corrected}$ = 0.015; OR 2.48) of 3'UTR +12197 polymorphisms were observed among female patients with PTB as compared to female HCs.

Conclusion: The study suggests that G/A genotype of In2+5887 and G/G genotype of 3' UTR+121973 polymorphisms may be associated with susceptibility to PTB among females and haplotype G-C-A-T may be associated with protection in females.

Implications: The findings may be useful to understand genetic susceptibility and resistance to PTB.

(viii) Effect of vitamin D_3 on chemokine expression in PTB

(Principal Investigator: Dr.P. Selvaraj)

Background: Chemokines are a family of small cytokines with molecular weight of 8–10 kDa. They are responsible for the activation of monocytes, macrophages and other leucocytes. Vitamin D_3, induces antimicrobial peptide cathelicidin

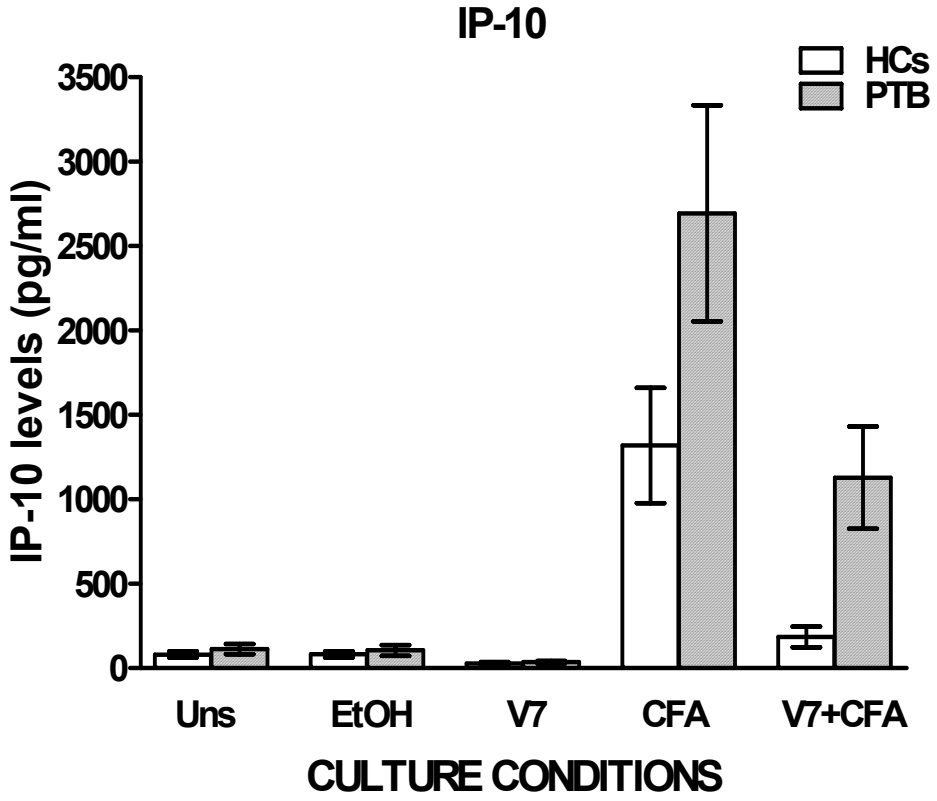
expression and increases cell migration and secretion of signaling molecules such as cytokines and chemokines from activated cells. *M. tuberculosis* infection of macrophages results in the induction of various chemokines that are required for the formation of the tuberculous granuloma and inhibition of its growth.

Aim: To find out the effect of vitamin D_3 on chemokine expression in PTB

Methods: The study was carried out in 20 PTB patients and 20 healthy control subjects. Peripheral blood mononuclear cells were cultured with live *M. tuberculosis* and its culture filtrate antigen (CFA) in the presence and absence of vitamin D_3 for 48 hrs. Chemokines such as Monocyte chemoattractant protein-1 (MCP-1), Macrophage inflammatory protein-1α and -1β (MIP-1α, MIP-1β), Regulated on activation, normal T- cell expressed and secreted (RANTES) and Interferon-γ IP-10 and Monokine induced by Interferon-γ (MIG) were estimated using commercially available cytometric bead array kit. Total RNA extracted from 48 hrs old macrophages was used for complementary DNA (cDNA) synthesis. The relative quantification for the target genes MCP-1, MIP-1α, MIP-1β, RANTES,IP-10 and house keeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was done using real time PCR (RT-PCR) with TaqMan assay primers and probes.

Results: In healthy control subjects, vitamin D_3 significantly suppressed the MCP-1 mRNA expression of CFA stimulated cells ($p=0.0027$), while no such effect was observed in PTB patients. Vitamin D_3 showed no significant effect on MIP-1α, MIP-1β and RANTES in both the study groups. However, the CFA induced IP-10 mRNA and protein expression were significantly suppressed by vitamin D_3 in both the study groups ($p<0.05$) (Fig. 10). A similar suppressive effect of vitamin D_3 was observed with MIG protein in healthy controls ($P=0.0029$) and a trend towards a suppression was observed in PTB patients.

Fig. 10: Effect of vitamin D_3 on IP-10 levels in PTB



Uns: Unstimulated cells; EtOH: Ethanol control; V7: Vitamin D3 at 1x10−7 M concentration; CFA: Culture filtrate antigen of *M.tuberculosis.*

Conclusion & Implication: The study suggests that vitamin D_3 may down regulate the recruitment and activation of T-cells through IP-10 and MIG chemokines at the site of infection and may act as a potential anti-inflammatory agent.

(ix) Role of pleural mesothelial cells in tuberculous immunity: **Expression of TLRs by non-phagocytic pleural mesothelial cells**

(Principal Investigator: Dr.D. Sulochana)

Background: Although pleural mesothelial cells (PMC) are not professional antigen presenting cells (APC), they express innate immune receptors like TLRs, probably to initiate innate defense mechanisms. The expression of TLR-2, -4 and -9 was evaluated in order to understand the role of PMC in innate immunity.

Further analysis of the TLR pathway triggered by *M. tuberculosis* was made by assessing the subunits of transcription factors AP-1 and NF-κB.

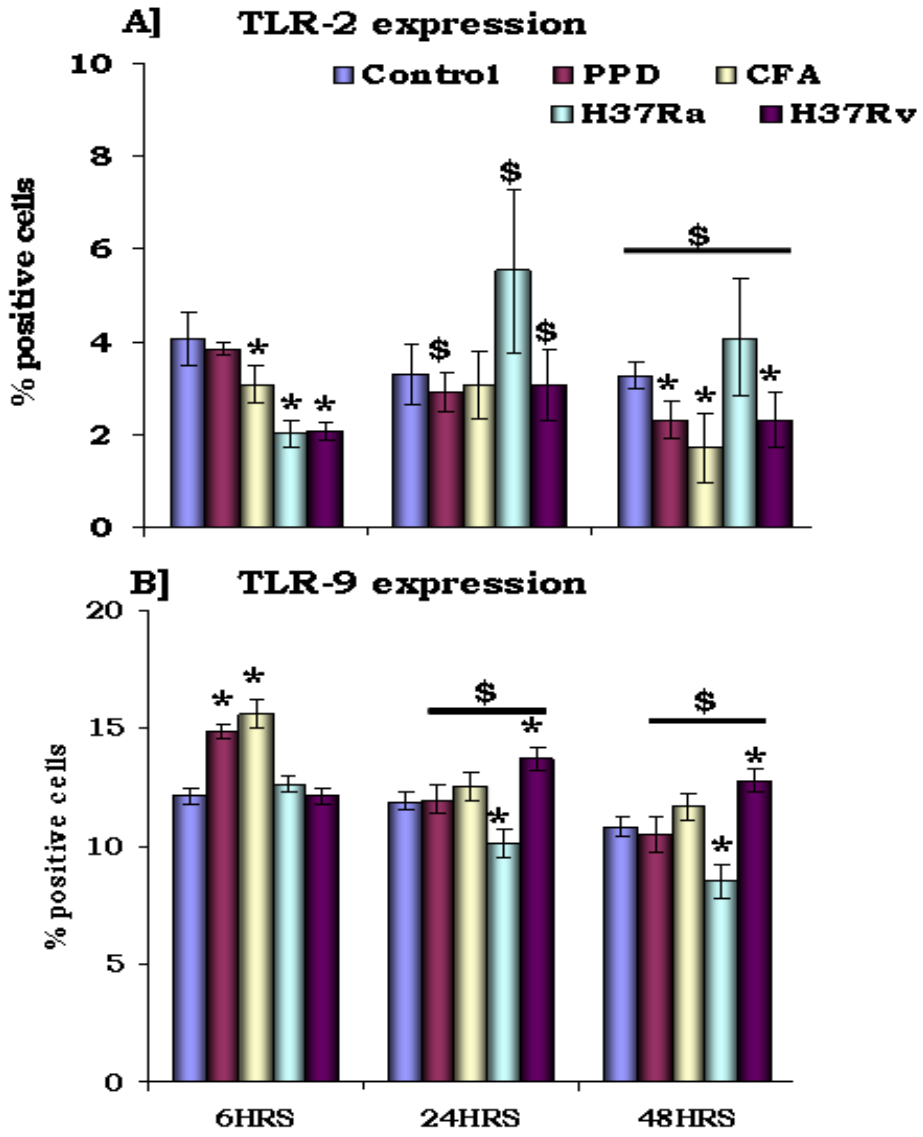
Aim: To study the expression of TLRs in infected PMC and its role in the pathogenesis of tuberculous pleurisy

Method: The transformed mesothelial cells (Met5A cell line) were infected with H37Ra and H37Rv and also stimulated with CFA and PPD. The cells were harvested at 6, 24 and 48 hrs time points and stained for the surface expression of TLR-2, -4 and -9. The nuclear transcription factors like NF-κB and AP-1 were evaluated in cell lysates using ELISA.

Results: There was no expression of TLR4 on Met-5A cells indicating that this receptor might not be expressed on human mesothelial cells. The expression of TLR-2 was significantly decreased at the early time point of 6hrs in infected PMC. However, H37Ra showed increased TLR2 expression at later time points when compared to respective controls. On the contrary, PPD and CFA increased the TLR-9 expression at early time point followed by H37Rv (Figs. 11A & B). The nuclear extract of the stimulated cells showed a significant decrease in c-Jun levels, a major component of the AP-1 transcription factor, at 24hrs in all culture conditions. An interesting pattern of c-Fos expression was observed: H37Ra and CFA proteins stimulated the expression of c-Fos at early time point whereas H37Rv induced its expression at later time point (Figs. 12A and B). The expression of NF-κB p50 was noticed at all time points but was not significantly altered. The expression of NF-κB p65 was increased at 24hrs in CFA and PPD stimulated cells (Figs. 13A & B).

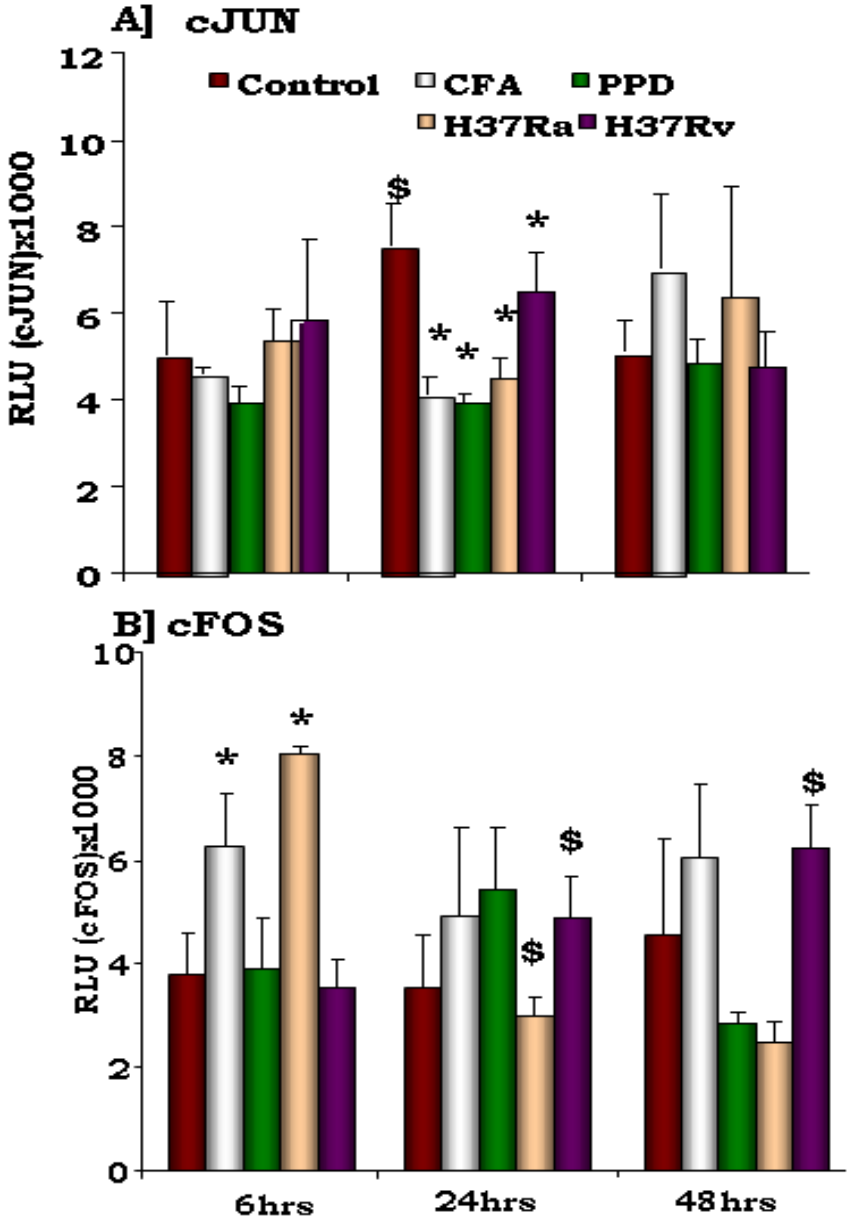
Conclusion: The human mesothelial cell line Met-5A expresses TLR2 and TLR9 but not TLR4. The constituents of the ManLAM from these strains might be the cause for the observed preferential up/down regulation of the studied TLRs.

Fig. 11: Expression of TLRs in *M.tb* or its antigen treated mesothelial cells



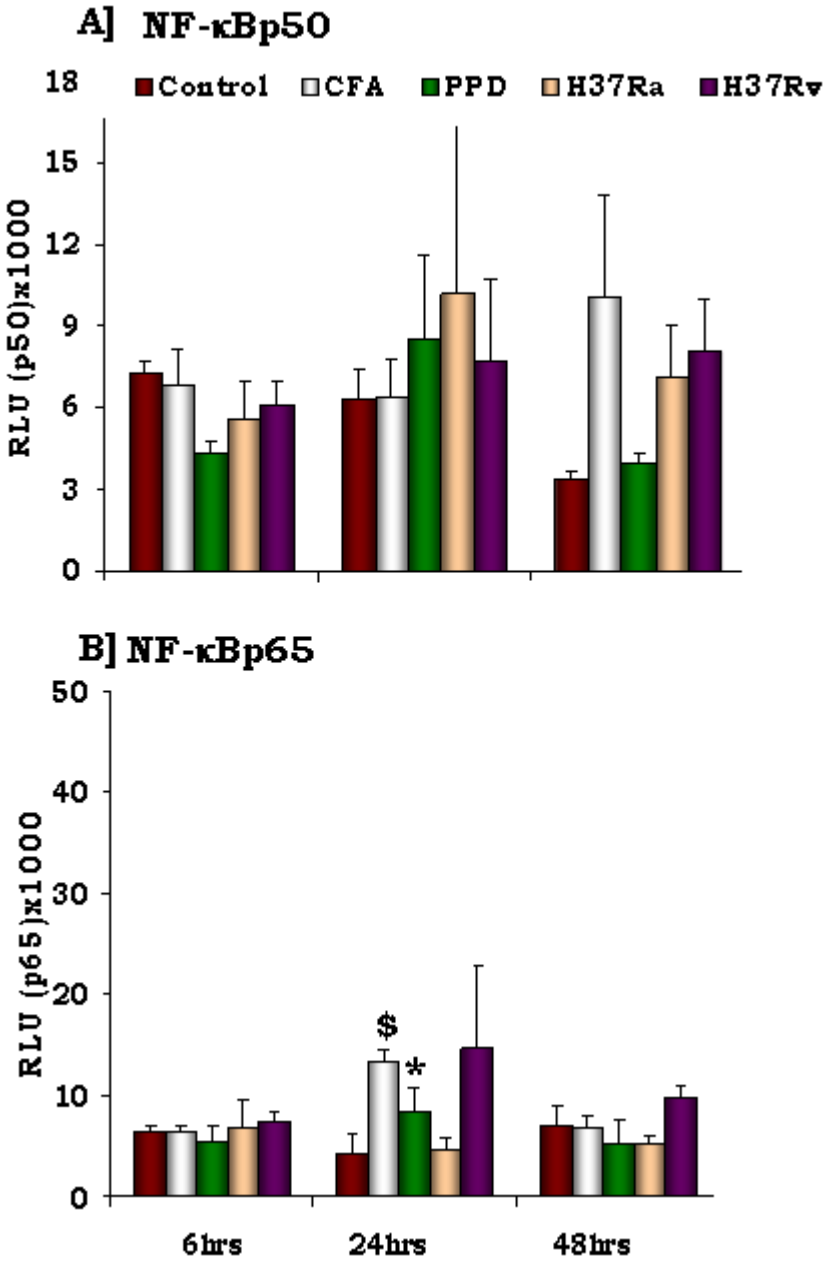
The expression of TLRs is presented as percentage positive cells in triplicates with mean ± SEM. $ represents $p<0.05$ compared to 6 hours, * represents $p<0.05$ compared with controls.

Fig. 12: Expression of c-Jun and c-Fos in *M.tb* or its antigen treated mesothelial cells at various time points



The levels of transcription factors expressed as relative light unit (RLU) in triplicates with mean ± SEM, $ represents $p<0.05$ when compared with the levels at 6hrs, * represents $p<0.05$ when compared with controls.

Fig. 13: Expression of p50 and p65 in *M.tb* or its antigen treated mesothelial cells at various time points



The levels of transcription factors expressed as relative light unit (RLU), in triplicates with mean ± SEM, $ represents $p<0.05$ when compared with the levels at 6hrs, * represents $p<0.05$ when compared with controls.

(x) Role of matrix metallo proteinases in tuberculous pleuritis

(Principal Investigator: Dr.D. Sulochana)

Background: *M. tuberculosis* infection induces the production of matrix metallo proteinases **(**MMPs) in macrophages leading to the breakdown of the extracellular matrix (ECM) which is necessary for cell recruitment and efficient granuloma formation. Thus, MMP production may be essential for the development of protective immune responses to mycobacteria. However, MMP activity may also play an important role in the pathology of mycobacterial infections by destroying the tissue integrity.

Aim: To study the levels of different MMPs (MMP-1, MMP-7 and MMP-9) involved in inflammatory responses were compared in plasma and pleural fluids of TB and non-TB (NTB) patients to understand the pathophysiology of TB at the site of infection

Methods: Blood (BL) and Pleural Fluid (PF) samples were collected from 44 patients, of which 32 had exudative pleural effusions with lymphocytic predominance (TB) and 16 had NTB etiology. MMPs were estimated by Flow Cytometric Multiplex arrays based on traditional ELISA.

Results: The levels of MMP-1 were significantly increased in TB PF compared to TB BL and NTB PF (Fig. 14). The MMP-7 and -9 levels were significantly high in BL compared to PF in both TB and NTB groups (Figs. 15 & 16). The comparison of MMP-9 levels in two groups showed significantly high levels in TB PF compared to NTB group (Fig. 16).

Conclusion: MMPs play a major role in inflammatory responses and possess all possible characters to act as a valuable biomarker. It is evident from this work that their levels are altered at the site of infection and also in the systemic circulation indicating its compartmentalization and role in normal physiologic processes.

Fig. 14: MMP-1 levels in plasma (BL) and pleural fluid (PF) of non-TB (NTB) and TB groups

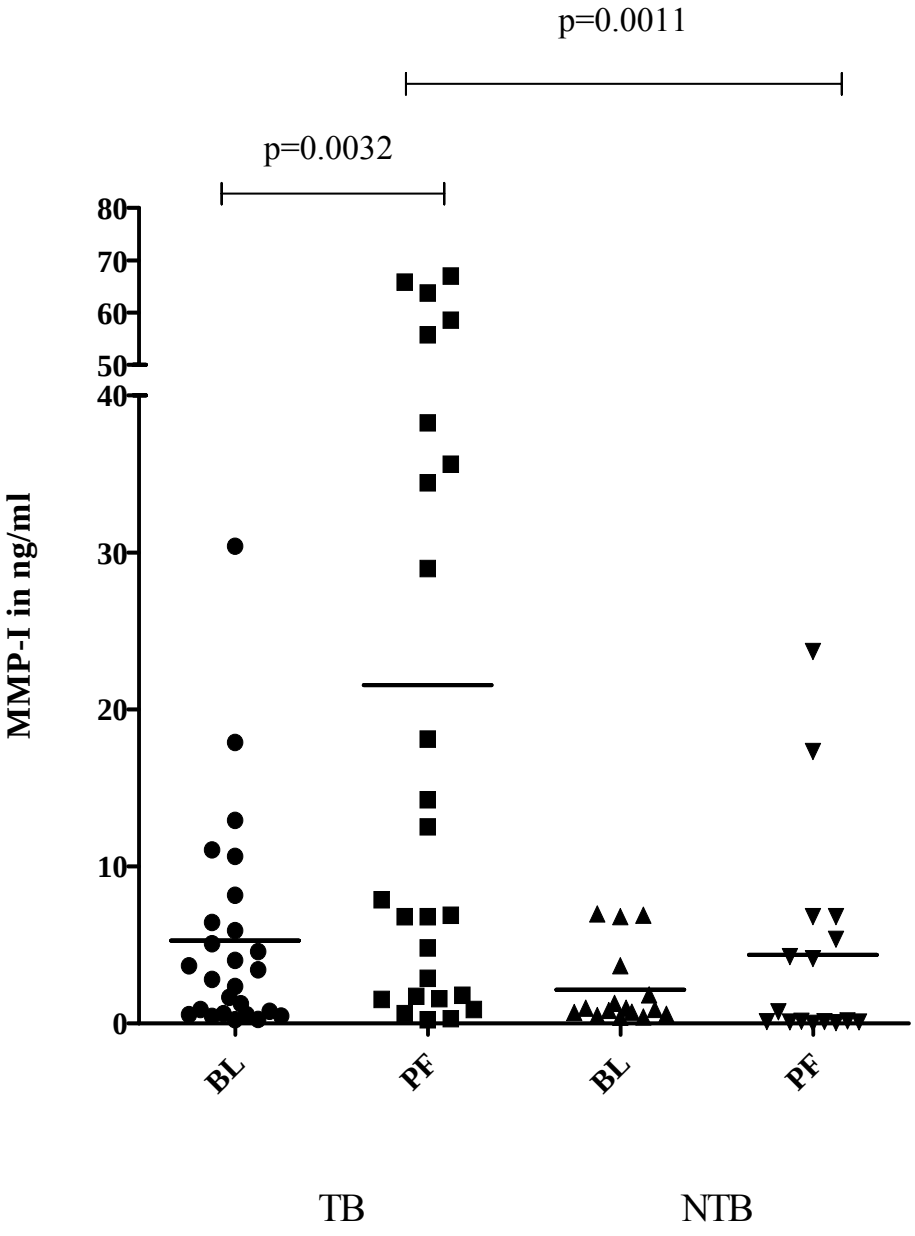


Fig. 15: MMP-7 levels in plasma (BL) and pleural fluid (PF) of non- TB (NTB) and TB groups

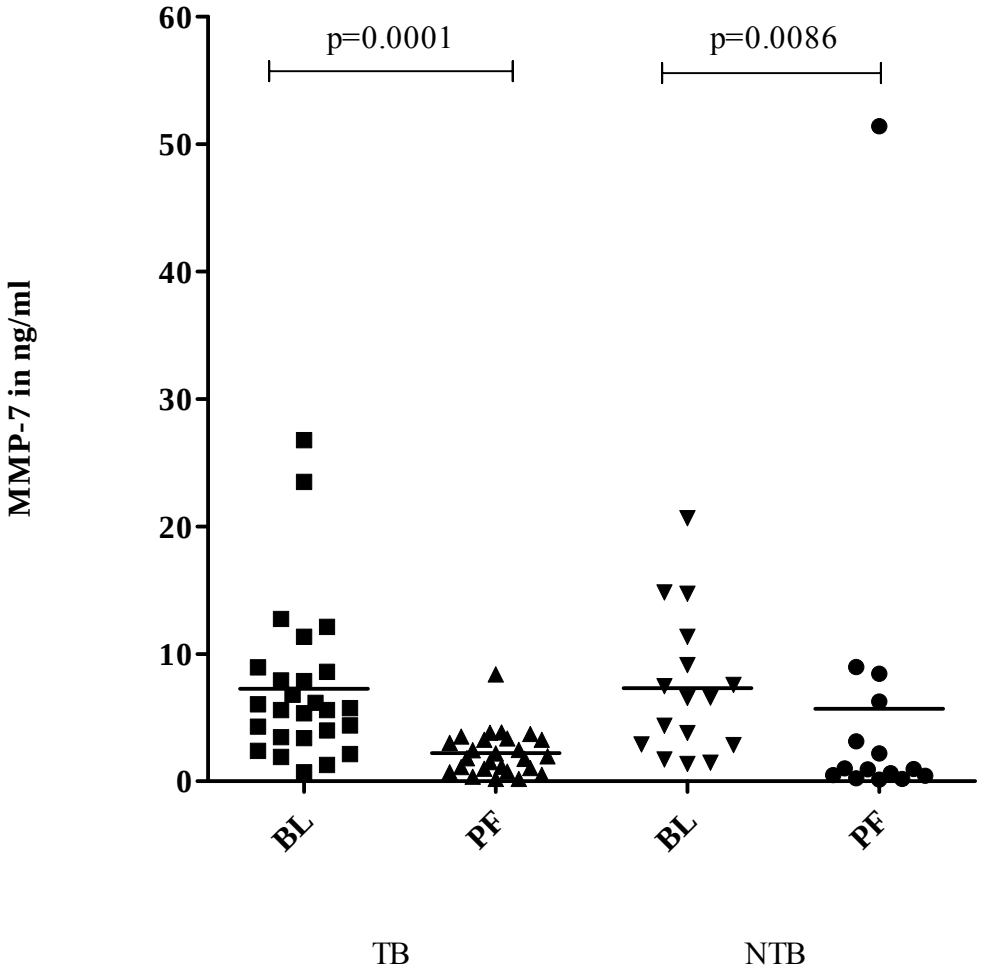
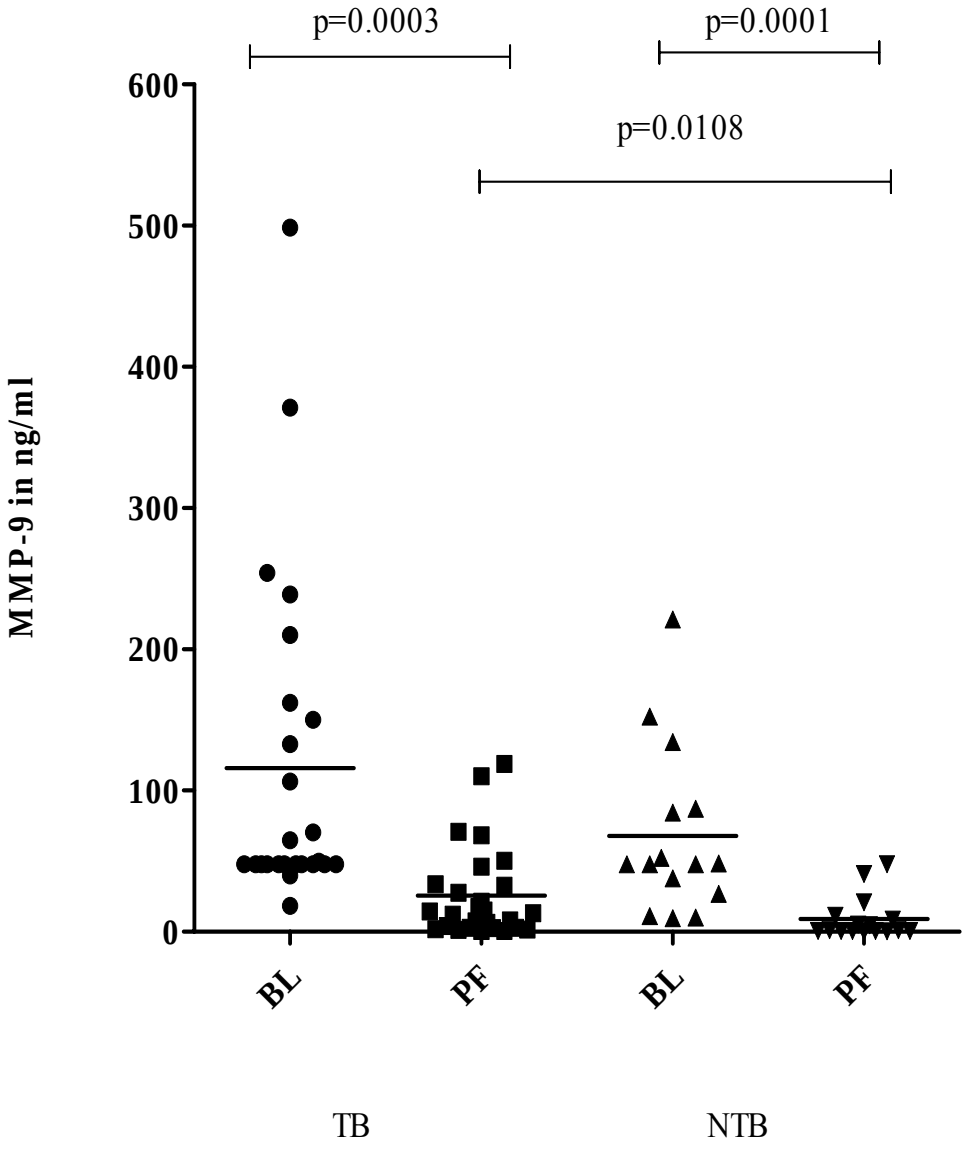


Fig. 16: MMP-9 levels in plasma (BL) and pleural fluid (PF) of non-TB (NTB) and TB groups



Studies in progress:

(i) Cloning, over-expression, purification and immunological characterization of novel T-cell antigen (PpiA) of *M. tuberculosis*

(Principal Investigator: Dr. Alamelu Raja)

Background: Earlier we had carried out systematic separation and analysis of *M. tuberculosis* culture filtrate proteins. PpiA was one of the novel T-cell antigens identified. Bioinformatics analysis also predicted PpiA protein to bind with more numbers of HLA alleles, compared with other novel T-cell antigens identified in our previous analysis. These factors prompted us to immunologically characterize the PpiA.

Aim: To immunologically characterize the recombinant PpiA protein of *M. tuberculosis*

Methods: PpiA gene is PCR amplified using H_{37}Rv genomic DNA as template. PCR amplified product was cloned in PrsetA vector. Cloned vector was transformed in *E. coli* BL21 (DE3) pLysS strain. PpiA protein was over expressed in transformed *E. coli* by IPTG induction.

Over expressed protein was purified by affinity chromatography and used in immunological analysis in 10 HCS (subjects who were apparently free of TB symptoms and did not have close family contact of TB) and 10 HC [Health care workers who were closely working with TB patients and apparently free from TB symptoms, those with latent TB infection (LTBI)]. Blood from the study subjects was diluted 1 in 10 with RPMI and was cultured along with PpiA protein (5µg/ml) and standard antigens (CFP-10 and Ag85B) (5µg/ml) for 6 days. After 6 days, the cell culture supernatant was collected and IFN-γ level was measured by ELISA.

Results: PpiA antigen induced significantly higher IFN-γ production in HC compared to HCS (Fig. 17). Smillar to PpiA, significant difference was observed in the other 2 standard antigens tested (CFP-10 and Ag85B) between HC and HCS (Fig. 18). By ROC curve analysis, and fixing a cut-off point, PpiA showed only 30% sensitivity for the prefixed 100% specificity.

Conclusion: In our analysis we found that PpiA antigen induced significantly higher IFN-γ response in HC (LTBI) compared with the HCS. However, this was

based on data from 10 subjects in each group. More study subjects will be included and data analysed.

Fig. 17: IFN-γ response against PpiA antigen

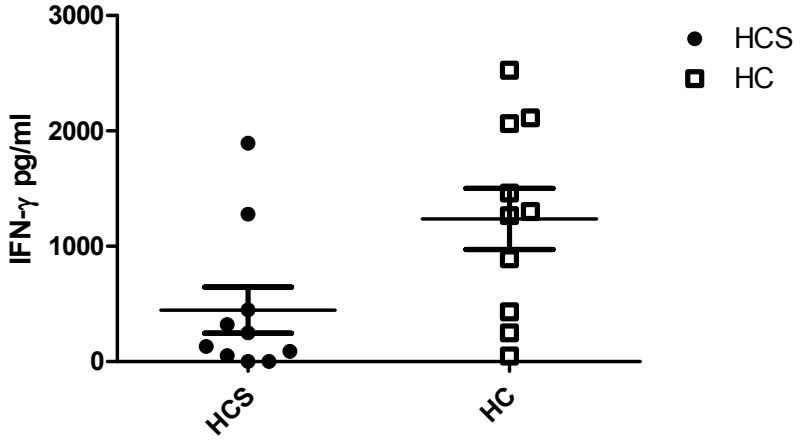
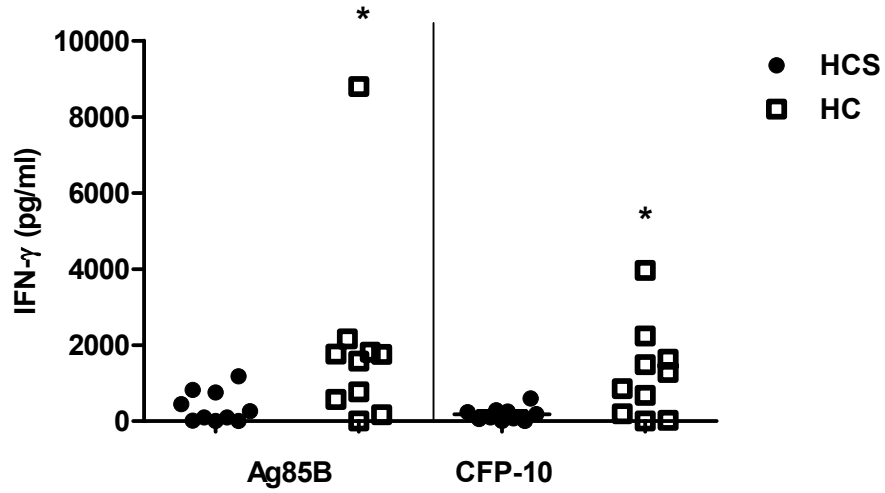


Fig. 18: IFN-γ response against Ag85B and CFP-10 protein



(ii) Humoral immune response to four recombinant antigens for serodiagnosis of TB

(Principal Investigator: Dr. Alamelu Raja)

Background: The detection of M. *tuberculosis* -specific human antibodies has been an important diagnostic aid in the diagnosis of TB. There is a heterogeneous humoral antibody response to antigens of *M. tuberculosis* (M. tb) among individuals so that the detection of antibodies against a single antigen usually has a low sensitivity for diagnosis of TB.

Aim: To determine the serodiagnostic potential of four recombinant antigens (38kDa, ADK, BfrB and MPT 64) among TB patients

Methods: Four recombinant *M. tuberculosis* antigens were assessed for their diagnostic potential in terms of sensitivity and specificity using indirect ELISA. Sera from 100 healthy control subjects (48 PPD –ve and QFT –ve), 80 PTB patients (54 S+ve C+ve, 26 S-ve C+ve, 28 cavity and 40 non-cavity), 108 healthy house hold contacts, 80 HIV positive subjects and 80 HIV-TB positive patients were studied.

Results: In PTB, the sensitivity of individual antigens ranged from 17% to 52.5% and the specificity was > 95%. When all four antigens were combined, the sensitivity was increased to 75% and the specificity was 88% (Table 9). ADK yielded sensitivities of 45.5% and 44.4% in both S+ve C+ve and S-ve C-ve HIV-TB group with >95% specificity in PPD –ve QFT –ve HCS. On combining all the four antigens, sensitivities increased to 54.5% and 55.6% in both S+ve C+ve and S-ve C-ve HIV-TB group respectively with a specificity of 88% (Table 10).

Conclusion: The 38kDa, ADK, BfrB, and MPT64 antigens can be used as cocktail antigens in the serodiagnosis of active TB. Since ADK shows more positivity in HIV-TB group, combination of antigens with ADK can be a diagnostic marker in HIV- TB.

Table 9: Combination of all antigens in five groups

	HCS, n = 100 (% positivity)	HHC, n = 108 (% positivity)	PTB, n = 80 (% positivity)	HIV, n = 80 (% positivity)	HIVTB, n = 80 (% positivity)
38kDa	3 (3)	15 (13.8)	42 (52.5)	6 (7.5)	23 (28.7)
MPT 64	4 (4)	16 (14.8)	29 (36.2)	7 (8.75)	22 (27.5)
ADK	4 (4)	10 (9.2)	19 (23.7)	15 (18.7)	21 (26.2)
BfrB	4(4)	6 (5.5)	17 (21.25)	11 (13.7)	14 (17.5)
38kDa + MPT 64 + ADK + BfrB	12 (12)	36 (33.3)	60 (75)	28 (35)	41 (51.2)

Table 10: ELISA positivity in smear positive and culture positive HIV TB group

	S+ve C+ve HIV TB		S-ve C-ve HIV TB	
	HCS, n = 48 (% Specificity)	HIVTB, n = 44 (% Sensitivity)	HCS, n = 48 (% Specificity)	HIVTB, n = 36 (% Sensitivity)
38kDa	2/48 (95.8)	10 (22.7)	2/48 (95.8)	13(36.1)
MPT 64	2/48 (95.8)	13 (29.5)	2/48 (95.8)	10 (27.8)
ADK	2/48 (95.8)	20 (45.5)	2/48 (95.8)	16 (44.4)
BfrB	2/48 (95.8)	5 (11.4)	2/48 (95.8)	8 (22.2)
38kDa + MPT 64	4/48 (91.6)	18 (40.9)	4/48 (91.6)	18 (50)
38kDa + ADK	4/48 (91.6)	22 (50)	4/48 (91.6)	19 (52.8)
38kDa + BfrB	3/48 (93.7)	12 (27.3)	3/48 (93.7)	15 (41.7)
MPT 64 + ADK	4/48 (91.6)	21 (47.7)	4/48 (91.6)	17 (47.2)
MPT 64 + BfrB	3/48 (93.7)	14 (31.8)	3/48 (93.7)	14 (38.9)
ADK+BfrB	4/48 (91.6)	21 (47.7)	4/48 (91.6)	16(44.4)
38kDa + MPT 64 + ADK	6/48 (87.5)	23 (52.3)	6/48 (87.5)	20 (55.6)
38kDa + MPT 64 + BfrB	4/48 (91.6)	19 (43.2)	4/48 (91.6)	20 (55.6)
38kDa + ADK + BfrB	5/48 (89.5)	23 (52.3)	5/48 (89.5)	19 (52.8)
MPT 64 + ADK + BfrB	5/48 (89.5)	22 (50)	5/48 (89.5)	17 (47.2)
38kDa + MPT 64 + ADK + BfrB	6/48 (87.5)	24 (54.5)	6/48 (87.5)	20 (55.6)

(iii) *In silico* prediction of T-cell epitopes from *M. tuberculosis* antigens (Principal Investigator: Dr. Alamelu Raja)

Background: An essential step towards the development of a new vaccine against TB is screening of *M. tuberculosis* antigens that elicit protective immune response. T-cell mediated immune response is critical for the development of resistance against mycobacterial infection. Thus mapping of antigenic peptide (epitope) sequences from proteins of tubercle bacilli recognized by T helper (Th) and by cytolytic T lymphocytes (CTL) is crucial for vaccine development.

Aim: To map antigenic peptides from 24 novel T-cell antigens of *M. tuberculosis* by using experimentally proven bioinformatics algorithm

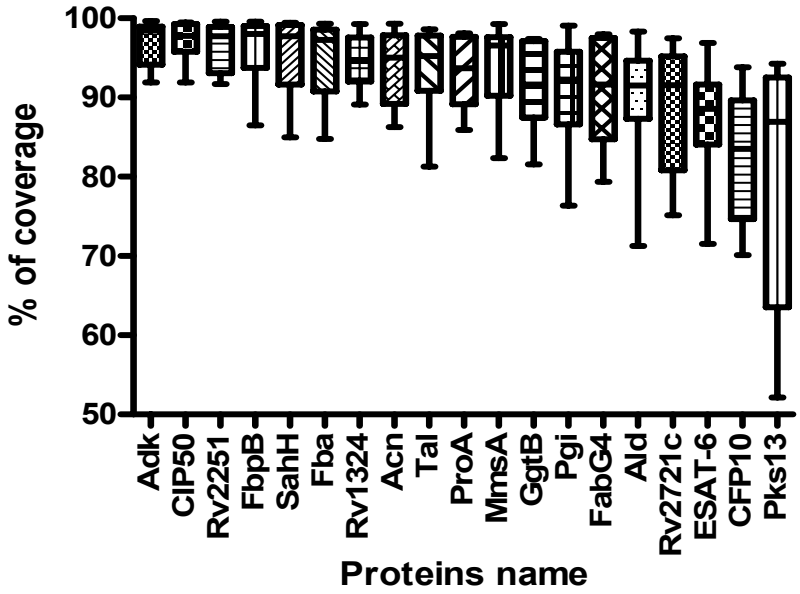
Methods: We have chosen 24 novel T-cell antigens that elicited IFN-γ response and mapped for both class I and II MHC epitopes using prediction software (Propred). In the design of peptide-based vaccines and diagnostics, the issue of population coverage in relation to MHC polymorphism is important, because of the fact that different HLA types are expressed at dramatically different frequencies in different ethnicities. Thus we have calculated population coverage for each of the selected proteins by IEDB population coverage calculation.

Results: The immunodominant regions of 24 novel T-cell antigens were predicted by submitting their FASTA sequences to Propred. Percentage of binding was calculated by proportion of alleles a protein binds to that of total number of alleles. Percentage of binding affinities of majority of the proteins was significantly greater than control proteins (ESAT-6, CFP-10 and Ag85B). Adk, CIP50, Rv2251c, Rv3248c, Fba, Rv1324, Acn, Tal, ProA, MmsA, Rv2394, Pgi, FabG4, Ald, Rv2721c and Pks13 having high binding affinity (> 90%) to both class I and II allele.

The percentage of coverage of each protein was higher than immunodominant and validated reference antigens (ESAT-6, CFP-10 and Ag 85B protein), except Pks13 (Fig. 19). Maximum population coverage rate (97.24%) was observed in Adk followed by CIP50 (96.9%). This suggests that these antigens may probably induce protective immune response in majority of the population when administered.

Conclusions: In the present study, four promiscuous putative epitopes viz., 67 VVLLWSPRS, 42 VVGVTTNPS, 178 MRFLLSAKS and 842 IRLMALVEY from human T- cell antigens of *M. tuberculosis* have been computationally validated as potential vaccine candidates across diverse ethnicities. Further *in vitro* experiments are required to ascertain the immunogenicity of these putative epitopes for designing effective vaccines against TB infection.

Fig. 19: Percentage of coverage calculated for each ethnicity per antigen



(iv) Uncovering new signaling proteins for cell division gene cluster of *M. tuberculosis*

(Principal Investigator: Dr. Sujatha Narayanan)

Background: The cell wall structure of *M. tuberculosis* deserves special attention because it is unique among prokaryotes, and it is a major determinant of virulence for the bacterium. The unique cell wall is rich in drug targets. The cell wall core consists of a peptidoglycan layer, a mycolic acid layer and an arabinogalactan polysaccharide connecting them. The detailed structure of the cell wall core is largely, although not completely understood. Protein-protein interactions play significant roles in many biological processes, such as in the

assembly of molecular complexes or in signal transduction. To achieve proper cell division, numerous cell division proteins interact with each other which lead to the septum formation. *In silico* analysis of the STPKs revealed PknI as part of a cluster of genes that includes DacB2, Ffh and FtsY. These play a role in the cell division of *M. tuberculosis*. Identifying protein-protein interactions is important to understand its role in cell division and drug designing.

Aim: To identify the interacting partners PknI and DacB2 of *M. tuberculosis* among cell division genes

Results:

Protein expression: PknI and DacB2 of *M. tuberculosis* were previously cloned in pRSETb vector and transformed in *E. coli* GJ1158 and *E. coli* BL21 cells respectively. The constructs were revived and over-expressed in LB broth with 1µl/ml. Carbenicillin (50mg/ml) and 0.3M NaCl (1µl/ml), 100mM IPTG (1µl/ml) were added as inducers. The cells were grown for further 4hrs at 37^{o}C and pelleted down by centrifugation at 8,000 rpm for 10 min at 4^{o}C.

Protein Purification: The over-expressed PknI and DacB2 cells were purified using nickel affinity chromatography and His-tag recombinant proteins were eluted using pellet lysis buffer containing 200mM imidazole. The purified proteins were visualized in SDS-PAGE and confirmed with Western blotting. PknI protein and DacB2 were purified at 75kDa and 110kDa resoectuvekt and correlated with their molecular weight.

Protein-protein interaction: To investigate the protein-protein interaction, the cytoplasmic and crude membrane proteins of *M. tuberculosis* were used as prey proteins and the recombinant PknI and DacB2 were used as bait proteins.

PknI protein interactions: In order to identify the PknI interacting proteins using Far-western blotting, the prey proteins were separated using SDS-PAGE and Tricine-PAGE (For identifying low and high molecular weight proteins). PknI interacting proteins were then identified by adding recombinant His tagged PknI. Bands around ~65kDa and ~45kDa with both cytoplasmic and crude membrane proteins were identified as PknI interacting proteins. This interaction was further confirmed by electro eluting the interacting proteins and again binding with

recombinant PknI (Figs. 20a & b). Thus the novel proteins interacting with *M. tuberculosis* PknI were found to be specific.

Figs. 20 a & b: PknI interacts with *M. tuberculosis* proteins

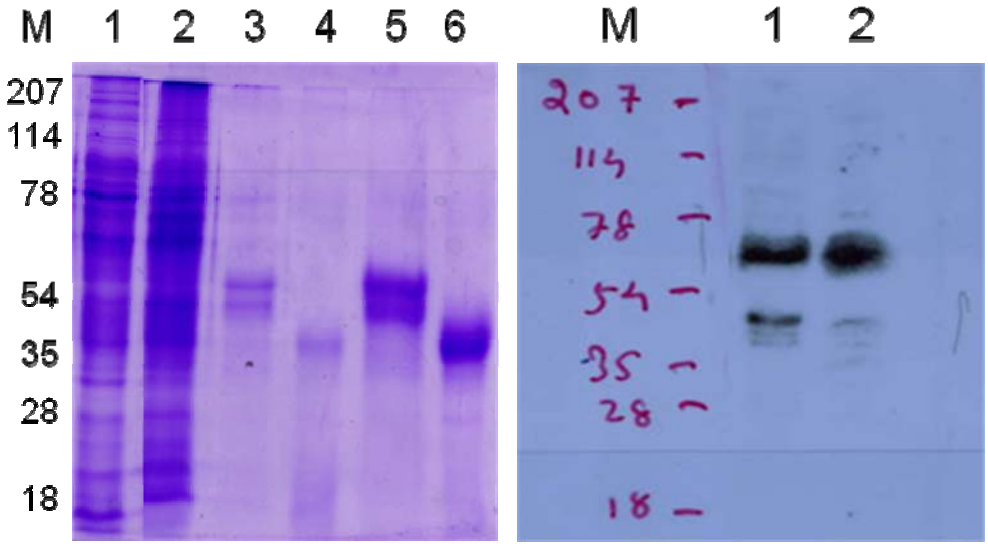


Fig. 20a: Lane1 cytoplasmic proteins, Lane2 H37Rv cure membrane proteins, Lane 3 to 6 Electro-eluted proteins. Fig. 20b: Lane1 H37Rv cytoplasmic proteins, Lane2 H37Rv crude membrane proteins.

DacB2 Protein interactions: The interacting proteins for DacB2 were also identified similar to that of PknI. Bands around ~55kDa, ~50kDa, ~40kDa with cytoplasmic proteins and ~30kDa, ~28kDa and ~20kDa with crude membrane proteins were identified as *M. tuberculosis* DacB2 interacting proteins (Figs. 21 a & b).

Figs. 21a & b: DacB2 interacts with *M. tuberculosis* proteins

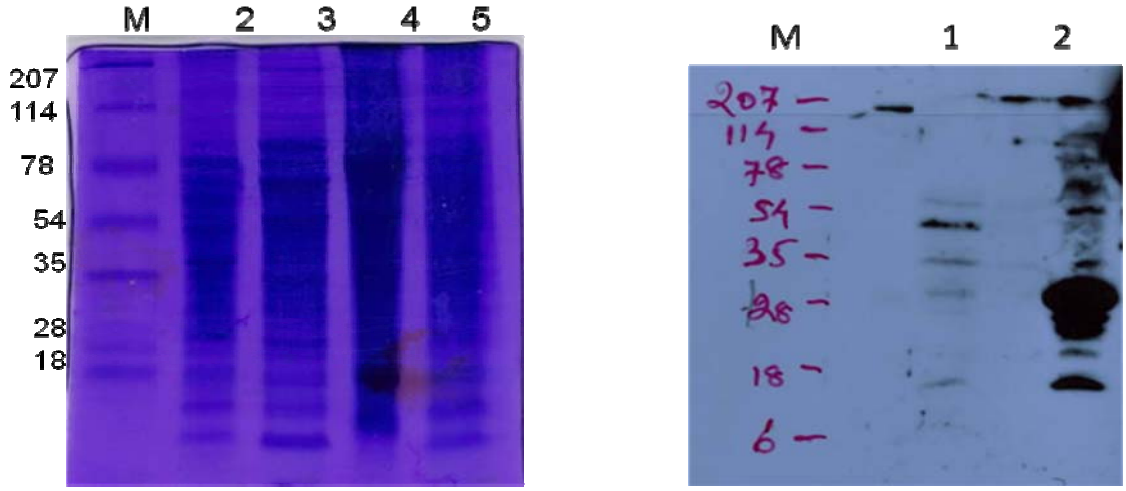


Fig. 21a: Lane 2 H37Rv cytoplasmic proteins, Lane 3 H37Rv crude membrane proteins, Lane 4 JalH37Rv cytoplasmic proteins, Lane 5 JalH37Rv crude membrane proteins. Fig. 21b: Lane 1: H37Rv cytoplasmic proteins; Lane 2: H37Rv crude membrane proteins.

Conclusion: The full length pknI and dacB2 genes have been over-expressed and affinity purified. Preliminarily, we have found that the interacting proteins for PknI and DacB2 were present in both cytoplasmic and crude membrane fractions of *M. tuberculosis* H37Rv. Characterizing the identified substrates will be helpful in understanding the signaling mechanism used by these proteins in cell division of *M. tuberculosis*.

(v) PknE, a serine/ threonine protein kinase is involved in modulation of MAPK signaling

(Principal Investigator: Dr. Sujatha Narayanan)

Background: *M. tuberculosis* persists in long-term chronic infection, harboured within macrophages. The strategies for survival employed by *M. tuberculosis* long remains an attractive field of study. Functional genomics of *M. tuberculosis* revealed the presence of 11 serine/threonine protein kinases (STPKs) besides the 11 two component systems that play a major role in the pathogenesis of TB. Previous studies on *pknE*, a STPK from our lab showed its prominence in suppressing apoptosis in response to nitric oxide stress of the host Mitogen-Activated Protein Kinases (MAPKs) are evolutionarily conserved signal transduction pathways that play important roles in the transduction of signals in

the innate immune responses of plants, insects, and mammals. Besides this, MAPK signaling also plays a role in survival responses. In view of the role of PknE in influencing the inhibition of apoptosis, we further wanted to examine its role in modulating MAPK signaling.

Aim: To examine the dynamics of MAPK (p38MAPK, Erk1/2 and SAPK/JNK) phosphorylation between the wildtype H37Rv and Δ*pknE* (deletion mutant of pknE)

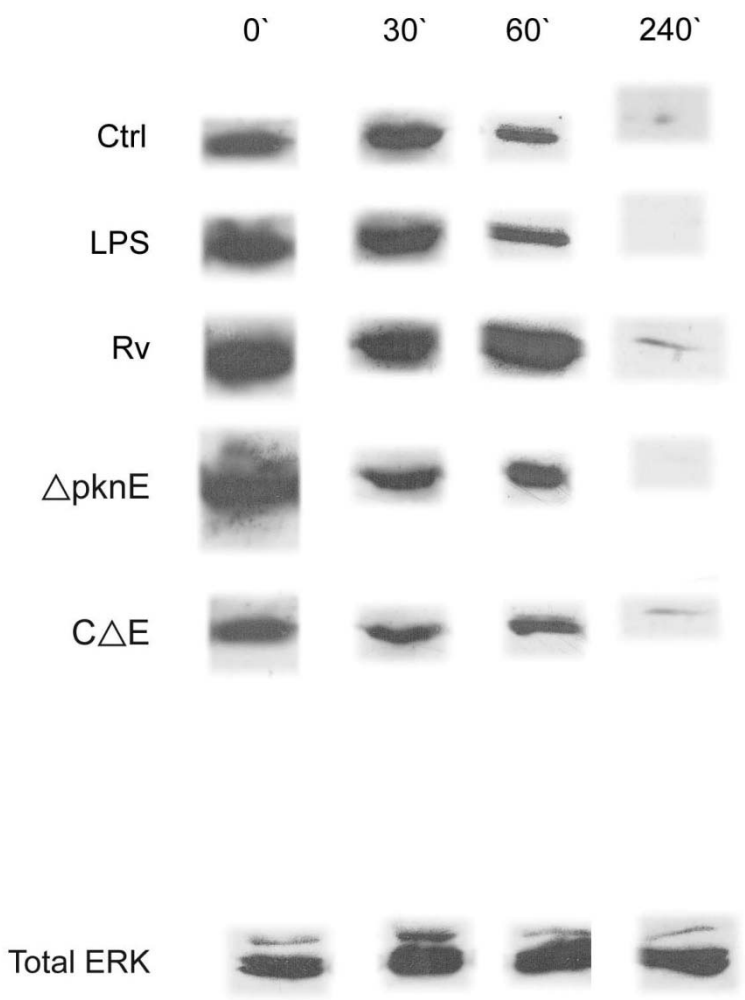
Methods: *M. tuberculosis* H_{37}Rv (wild-type), *M. tuberculosis* H_{37}Rv*pknE*::hyg (Δ*pknE*) and complemented *M. tuberculosis* H_{37}Rv*pknE* strains (CΔE) were grown in Middlebrook complete 7H9 broth and single cell suspensions were made from mid-exponential phase cultures and the viability of the cells were determined using Middlebrook 7H10 agar plates. Differentiated THP-1 cells were infected at MOI 1:10 with respective strains. Post infection at varied time periods cell were lyzed using 100µl of 2X lysis buffer (125mM Tris (pH 6.8), 4% SDS, 20% glycerol, 100mM DTT, and 0.05% bromophenol blue). The lysates were denatured at 95^0C for 5 min, separated by 12% SDS polyacrylamide gel electrophoresis, transferred and blocked using 5% skim milk in TBS containing 0.1% Tween 20 (0.1% TBST) for 1h at room temperature. After two washes in TBST, the membrane was incubated overnight at 4^0C with rabbit anti-human polyclonal antibodies against phospho and nonphospho p38MAPK, Erk½ and SAPK/JNK (1:1000). After three washes in PBST, the membranes were incubated for 1 hr at room temperature with horse radish peroxidase-conjugated goat anti-rabbit antibody (1: 300 in TBST). After three washes in TBST, bound antibodies were then detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) by exposure to Eastman Kodak X-ray film.

Results: The phosphorylation kinetics of p38MAPK, Erk½ and SAPK/JNK was analyzed for controls [Ctrl (control) and lipopolysaccharide (LPS)] and *M. tuberculosis* strains [Rv (H37Rv), Δ*pknE* (deletion mutant of *pknE*) and CΔE (complemented Δ*pknE*)] in THP-1 derived macrophages. Rv infected macrophages increased the phosphorylation of Erk½, p38MAPK, and SAPK/JNK from 30 min post infection compared to the controls (Figs. 22, 23 & 24). In

contrast, Δ*pknE* infected macrophages abrogated the phosphorylation of Erk½ at 240 min (Fig. 22), reduced the phosphorylation of p38MAPK at 60 min (Fig. 23), and selectively inhibited the phosphorylation of p46 subunit of SAPK/JNK at 120 min (Fig. 24) post infection compared to Rv infected macrophages. CΔE was able to partially restore the phosphorylation event observed in Rv. Use of specific inhibitors to MAPKs did not suppress the phosphorylation events in Δ*pknE* infected macrophages; instead a crosstalk between p38MAPK and Erk½ signaling was observed (Fig. 25).

Conclusion: The data from our study suggests that *pknE* has a role in the modulation of intracellular signaling that favors the survival of *M. tuberculosis*.

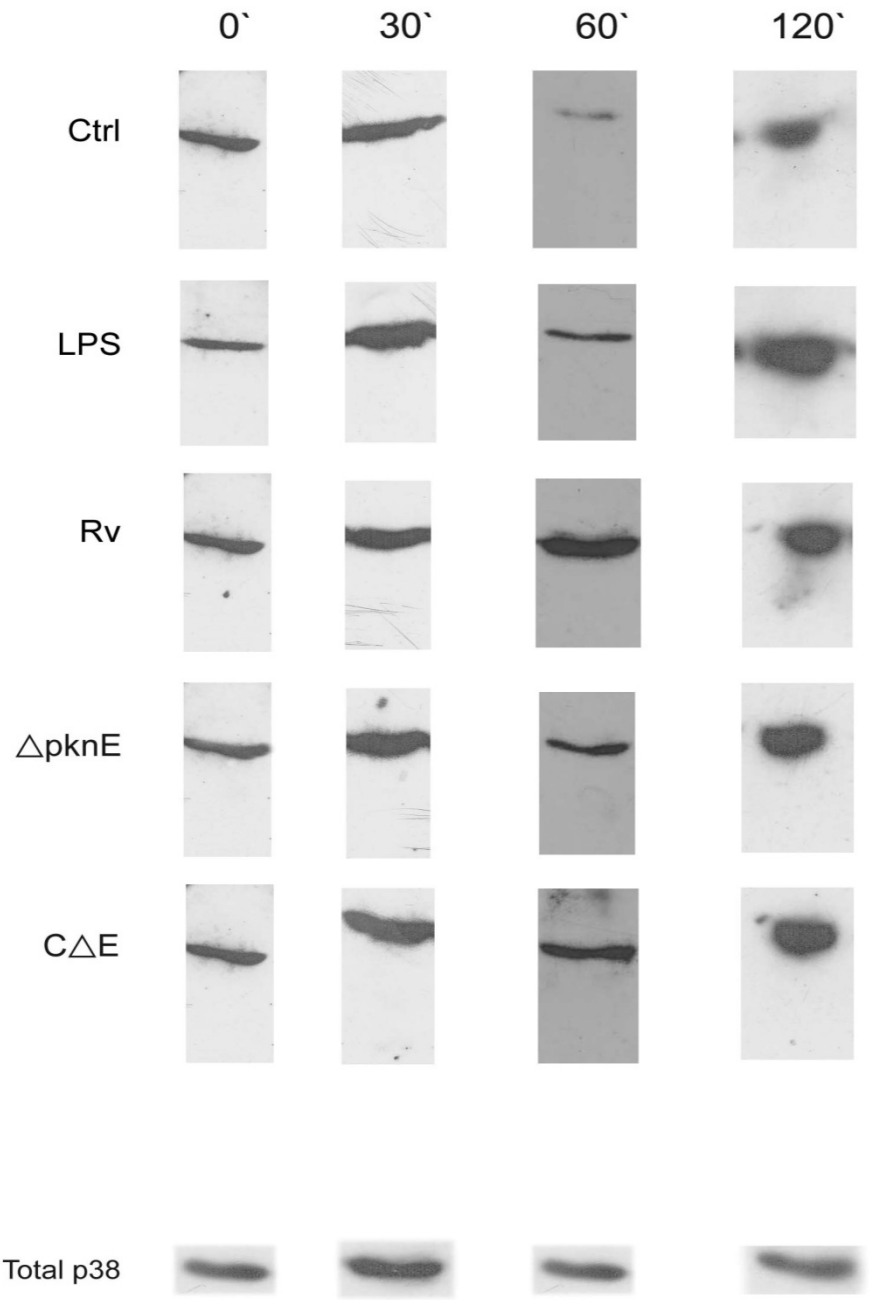
Fig. 22: Western blot showing phosphorylation kinetics of Erk½



Controls and infected cells were lysed post infection at varied periods and subjected to western blotting.

The blots were probed with phospho and non phospho Erk½ antibodies Results are from three independent experiments. Total Erk½ is used as loading control. Ctrl denotes control, DMSO denotes dimethylsulphoxide and LPS denotes lipopolysaccharide.

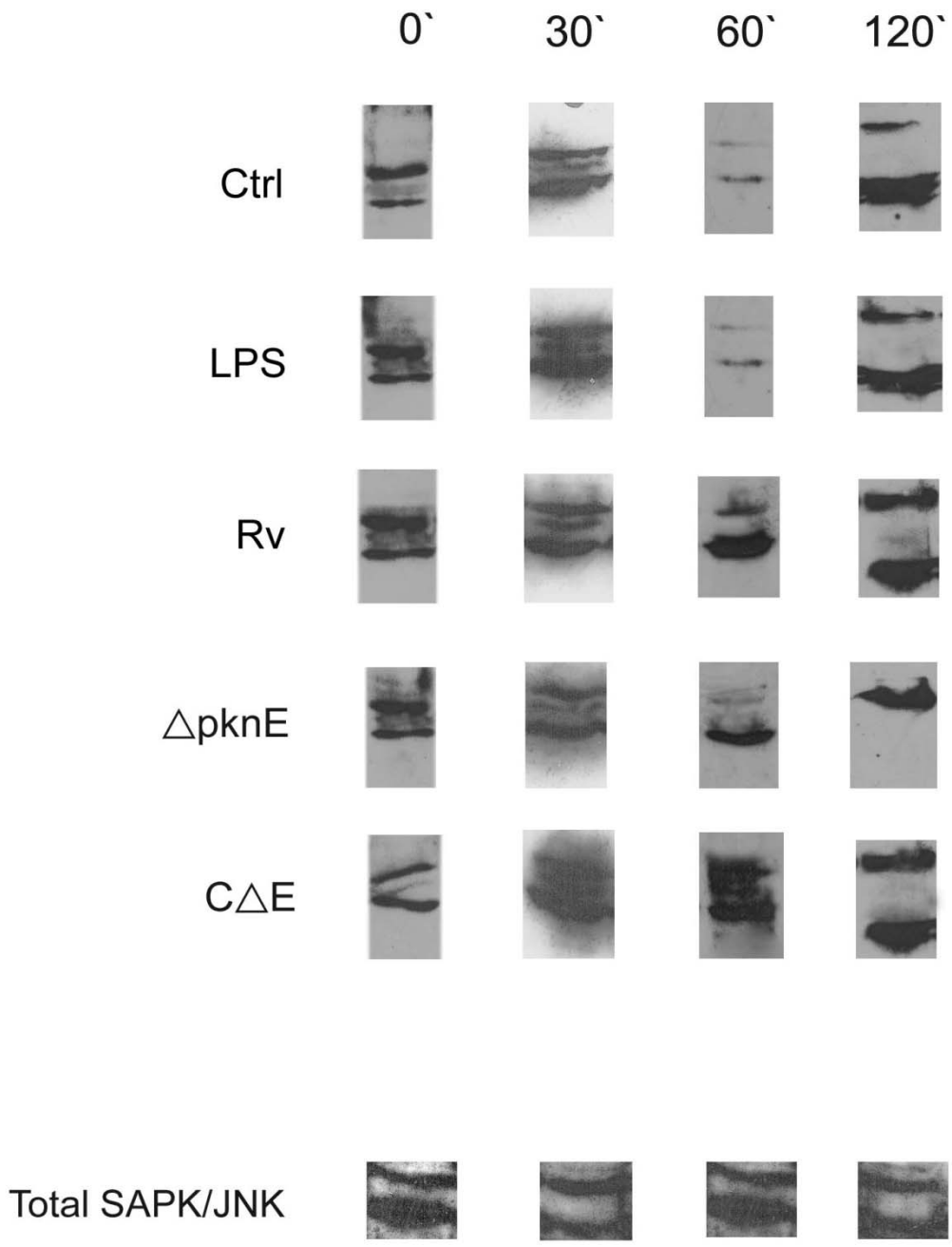
Fig. 23: Western blot showing phosphorylation kinetics of p38



Controls and infected cells were lysed post infection at varied periods and subjected to western blotting.

The blots were probed with phospho and non phospho p38 antibodies. Results are from three independent experiments. Total p38 was used as loading control. Ctrl denotes control, DMSO denotes dimethylsulphoxide and LPS denotes lipopolysaccharide.

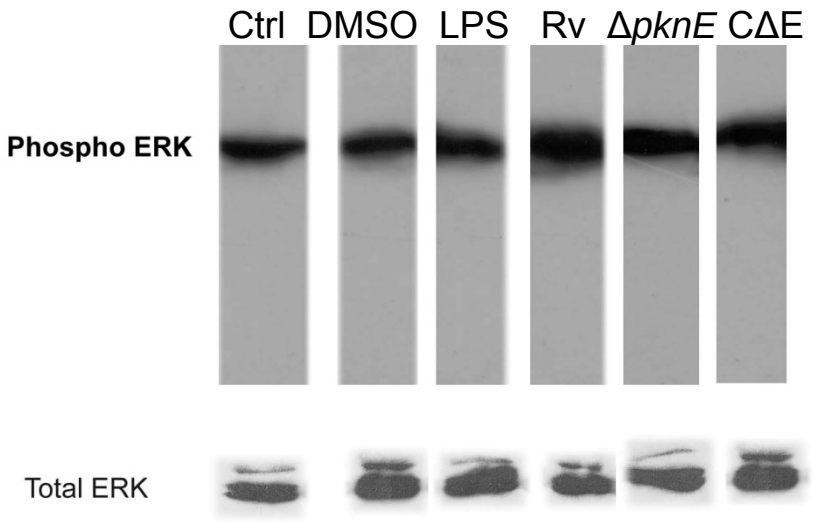
Fig. 24: Western blot showing phosphorylation kinetics of SAPK/JNK



Controls and infected cells were lysed post infection at varied periods and subjected to western blotting.

The blots were probed with phospho and non phospho SAPK/JNK antibodies. Results are from three independent experiments. Total SAPK/JNK was used as loading control. Ctrl denotes control, DMSO denotes dimethylsulphoxide and LPS denotes lipopolysaccharide.

Fig. 25: Crosstalk analysis between p38 and Erk½ pathways



Cells to be infected were treated with p38 inhibitor for 1 h and lysed 1 h post infection. The lysates were subjected to western blotting and probed with phospho and non phospho antibodies. The results are from three independent experiments. Total ERK was used as loading control. Ctrl denotes control, DMSO denotes dimethylsulphoxide and LPS denotes lipopolysaccharide.

(vi) Cloning, overexpression and construction of deletion mutant of LPQS of *M. tuberculosis*

(Principal Investigator: Dr. Sujatha Narayanan)

Background: Lipoproteins constitute 2.5% of the *M.tuberculosis* proteome. LpqS is a putative lipoprotein of *M. tuberculosis* which does not have any homolog outside slow growing mycobacteria and is implicated in enduring hypoxic response. Hence Lpqs might be essential for adaptation to hypoxic conditions.

Aims: (i) to clone and overexpress the lipoprotein lpqs of *M.tuberculosis* and

(ii) to characterize the function of the gene by the deletion of lpqs gene in *M.tuberculosis*

Materials and methods: Expression of *M.tuberculosis* LpqS in *M.smegmatis: Lpqs* gene was PCR amplified using suitable primers and cloned behind the inducible acetamidase promoter in the *E.coli* mycobacterium shuttle vector pMac206 using *E.coli* DH5α as the expression host. Recombinants were then screened and electroporated into *M.smegmatis*. Colonies carrying the

recombinant constructs were then checked for the expression of the lipoprotein lpqs by western blotting against his-tag (Figs. 26 & 27)

Construction of *M.tuberculosis LpqS* knockout mutants

Construction of allelic exchange substrate AES0847

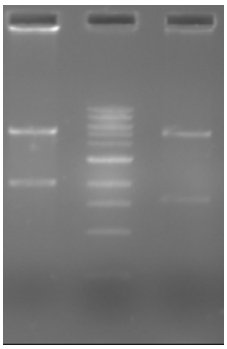
LpqS gene disruption was carried out using specialized transduction. The left and rights arm of the gene to be disrupted were PCR amplified using genomic DNA as template and suitable primers. Four fragment ligation was then carried out using Hygromycin-sacbB (3.6Kb) and ori segments (1.6Kb) of the p0004-sacB vector and the left and right arms of the gene to be disrupted to generate the allelic exchange substrate (AES) (Fig. 28). AES phasmid was constructed by sub-cloning AES0847 plasmids into temperature-sensitive TM4 phage Phae159 DNA followed by in-vitro packaging (Fig. 29). *E.coli* HB101 cells were used for transduction. AES Phasmid was then elctroporated into *M.smegmatis* to generate high titre phage.

Phage transduction: Targeted *M.tuberculosis* culture was grown and centrifuged to pellet the cells and the pellet was washed and resuspended in 1ml of the MP buffer. High titre phages prewarmed to 37^{o}C, mixed with equal volume of bacterial cell in MP buffer, incubated overnight at 37^{o}C. The mix was then centrifuged to pellet the cells, resuspended in 7H9 media and plated on 7H10 plates containing hygromycin for 4 to 5 weeks at 37^{o}C. Hygromycin resistant colonies were picked and screened for knockout mutants.

Confirmation of gene deletion: Gene disruption was confirmed by PCR using primers for hygromycin cassette and right flank (RR) (Fig. 30) and southern blotting using right arm as the probe. On probing, the wild type genome gave a band of 2.1kb and disrupted strain a band around 6.1kb (Fig. 31).

Complementation of knockout mutants: The knock-out strain of *Mycobacteria* was complemented by cloning *lpqs* behind the *hsp60* promoter in pMV361 (integrative backbone) vector and electroporating the construct into the mutant. Complementation of the mutant was confirmed by PCR.

Fig. 26: RED of lpqs cloned in pMac206

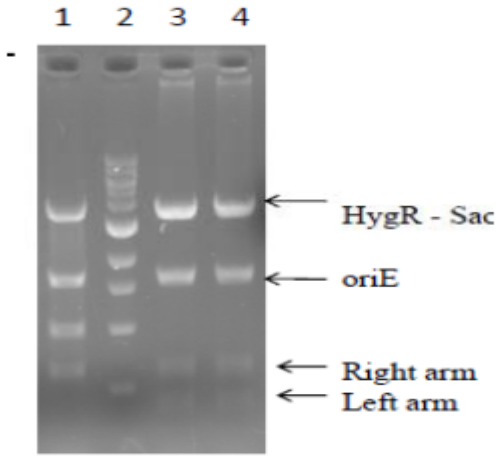


Lane1 : pMac206 lpqs
Lane 2 : 1kb ladder
Lane 3: pMac206

Fig. 27: Western blotting against his-tag to confirm the expression of lpqs in *M.smegmatis*

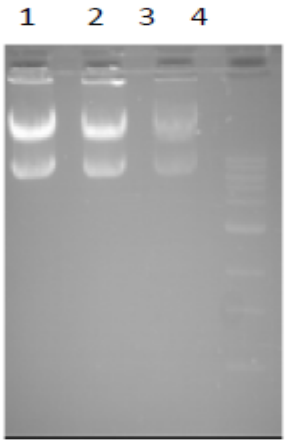
Lane 1 : Membrane fraction
Lane 2 : Soluble fraction

Fig. 28: Construction of AES



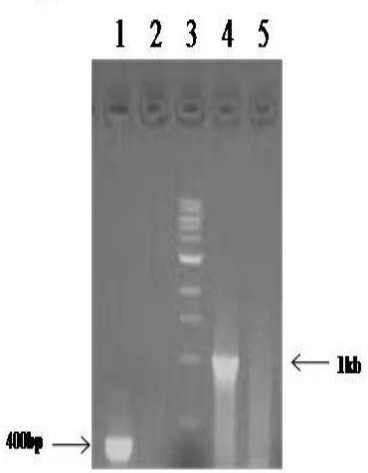
Lane 1: p0004-SacB
Lane 2 : 1 kb ladder
Lane 3&4: AES0847

Fig. 29: Construction of AES phasmid



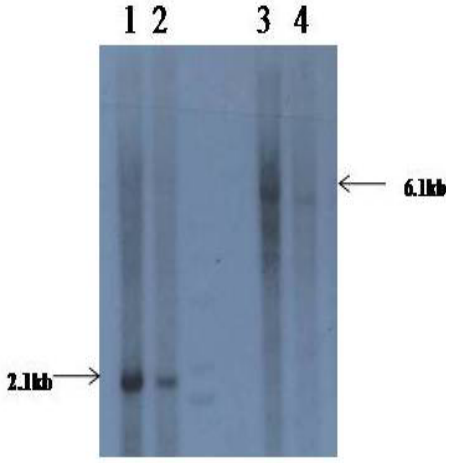
Lane 1,2 & 3 : AES phasmid
Lane 4 : 1kb ladder

Fig. 30: PCR confirmation of lpqs disruption in H37Rv



Genomic DNA of CΔ*lpqS* and Δ*lpqS* mutant as template for PCR an amplicon of around 400 bps was obtained for CΔlpqs and no product for mutant on using *lpqS* primers (lanes 1 and 2). A product arround 1 kb was obtained for the mutant and no product for H37Rv on using hyg^r forward and right arm reverse primer (lanes 4 & 5).

Fig. 31: Southern blot analysis of lpqs gene disruption in *M.tuberculosis* H 37Rv.



Genomic DNA from H37Rv and Δlpqs mutant was digesed with BamHI and ran on 1% agarose gel and blotted onto N^+ membrane. The blot was hybridised with right arm. The sizes were determined using the molecular DNA standards run in parallel.

(vii) Functional characterization of LPQS gene of *M.tuberculosis*

(Principal Investigator: Dr. Sujatha Narayanan)

Background: Lipoproteins of *M.tuberculosis* have been shown to be highly immunogenic proteins. Microarray analysis reveals a 4-fold increase in expression of LpqS gene under a non-proliferating anaerobic condition.

This lipoprotein might play an important role in the pathogenesis of *M. tuberculosis*.

Aims: (i) to carry out phenotypical characterization of the lpqs gene disrupted mutant ΔLpqS

(ii) to compare the intracellular viability of the mutant in macrophage model of infection

Methods:

Colony morphology and biofilm formation: Logarithmic phase cultures of H_{37}Rv, ΔLpqS and CΔLpqS strains were plated in serial dilutions on 7H10 plates containing SDS and copper.

***In vitro* growth kinetics:** Wild type H_{37}Rv, ΔLpqS and CΔLpqS cultures were grown to mid-log phase and culture OD adjusted to 0.5 with fresh media. Two sets of experiments one in 7H9 and another in sauton's media were carried out. Growth rate was assessed by plating the cultures in serial dilutions on 7H10 plates on day 3, 7, 10, 14 and 21. CFUs were counted after 4 to 5 weeks of incubation at 37°C.

Mammalian cell infection studies: Intracellular viability of the lpqs knockout mutants and the complemented strains were compared to wild type H37Rv using macrophage infection studies. THP1 cells were seeded onto 24 well plates (1 million cells/well) and differentiated into macrophages. Macrophages were then infected in triplicates with Wild type H_{37}Rv, ΔLpqS and the complemented strains at an MOI of 1:10. Wells were then washed with plain RPMI to remove the extracellular bacteria and incubated with fresh media in the carbon dioxide incubator. Adherent infected macrophages were then lysed and the lysates plated in serial dilutions on 7H10 plates to determine the viable intracellular

bacteria on days 2 to 7 after infection, CFUs were counted after 3 weeks of incubation at 37^{o}C.

Results: Successful deletion of *lpqs* from the genome of H37Rv showed that lpqs is a non-essential gene. Although LpqS mutant exhibited growth characteristics similar to H37Rv in nomal 7H9-ADS-T, it exhibited defective growth in sauton's minimal media compared to wild type. This clearly showed that mutant exhibits growth defect in nutrient deprived sauton's minimal media (Fig. 32). The mutant was found to be more susceptible to SDS (Fig. 33) and to increased concentration of copper *in vitro* compared to the wild type (Fig. 34). No obvious difference in colony morphology and biofilm formation was observed in the mutant compared to the parent strain. Disruption of lpqs in *M.tuberculosis* resulted in reduced intracellular survival of the mutant inside THP1 derived macrophages (Fig. 35). CFU of the mutant declined almost by 2 log compared to wild type H37Rv after day 7 of infection. Complementation of the mutant resulted in only partial restoration of the growth characteristics of the wild type.

Fig. 32: *In vitro* growth of wild type H37Rv, ΔlpqS and CΔlpqS

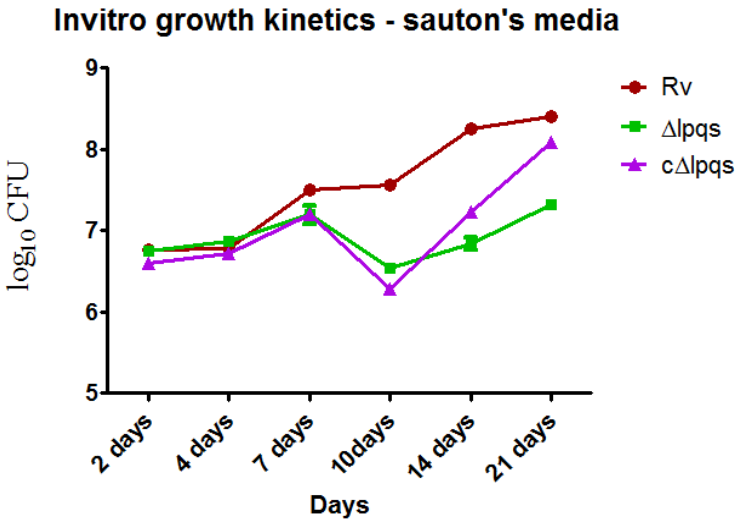


Fig. 33: Sensitivity of *M. tuberculosis* strains to SDS

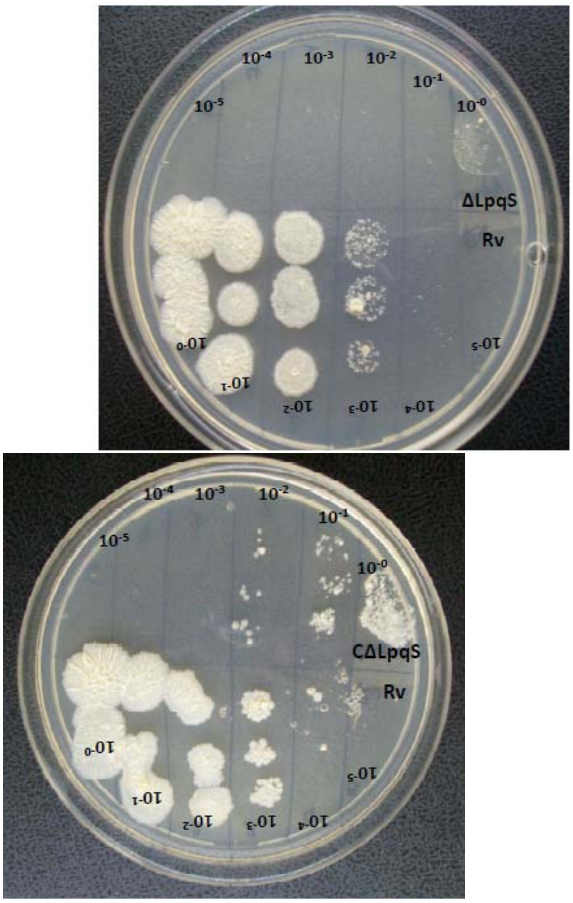


Fig. 34: Sensitivity of *M.tuberculosis* strains to copper

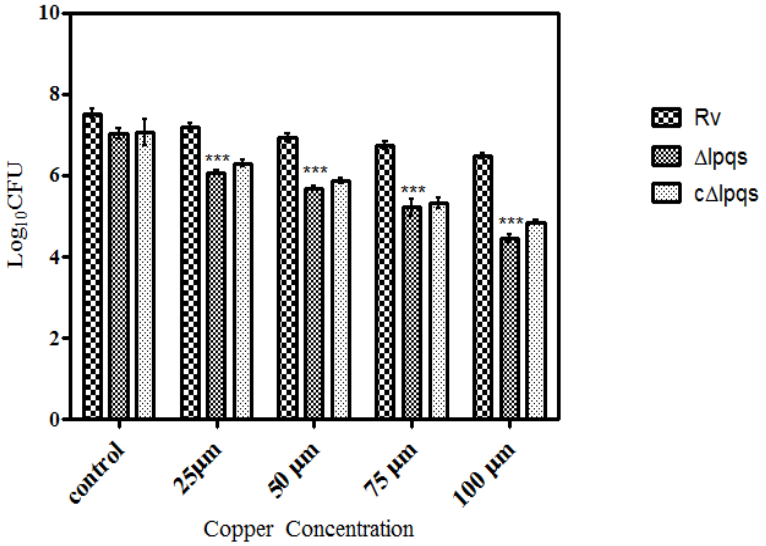
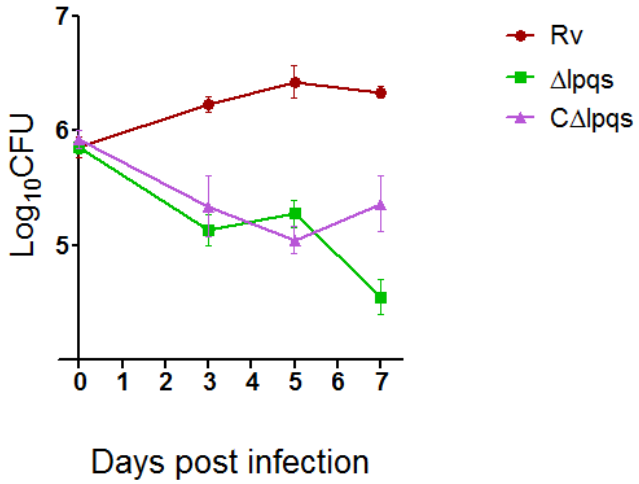


Fig. 35: Intracellular viability of ΔlpqS mutant compared to H37Rv



(viii) Role of Chemokine, DC-SIGN and TLR gene variants on immunity to TB

(Principal Investigator: Dr.P. Selvaraj)

Background: Invasion of the host by microbial pathogens causes activation of the innate immune response (first line defense) and triggers the secretion of various cytokines and chemokines and initiation of adaptive immunity. Chemokines along with cytokines are involved in the recruitment of T-cells to the inflammatory sites, activation of T-cells and inhibition of intracellular growth of *M. tuberculosis*. DC-SIGN (dentritic cell-specific ICAM-3 grabing nonintegrin), a C-type lectin, is the major *M. tuberculosis* receptor on human dentritic cells and involved in phagocytosis and cellular interactions. TLRs recognize lipid carbohydrate, peptide and nucleic acid structures expressed by various microorganisms. Polymorphisms of Chemokine, DC-SIGN and TLR genes have been shown to be associated with susceptibility or resistance to various infectious diseases.

Aims: (i) to find out whether chemokine, DC-SIGN and TLR gene polymorphisms are associated with susceptibility or resistance to TB (Part-I) and

(ii) to understand the role played by these gene variants on the innate and adaptive immunity to TB (Part-II)

Part-I of the study is carried out using stored DNA samples of 200 PTB patients and 200 healthy controls, collected earlier for various immunogenetic studies. Part-II of the study is carried out in a prospective manner using freshly drawn blood of 100 PTB patients and 100 healthy controls. Under this main study, the following studies are being carried out.

(ix) Regulatory role of chemokine gene polymorphisms on chemokine expression in PTB

(Principal Investigator: Dr.P. Selvaraj)

Background: Polymorphisms in the chemokine genes have been shown to regulate the production of chemokines. In the present study, the regulatory role of various chemokine gene polymorphic variants on chemokine expression is being studied in newly recruited PTB and healthy volunteers.

Aim: To understand the regulatory role of various chemokine gene polymorphisms on chemokine expression

Methods: The study is being carried out with newly recruited PTB and healthy volunteers. The chemokines such as MCP-1, MIP-1α, MIP-1β, RANTES, IP-10 and SDF-1 genes are being estimated in the 72h culture supernatants of peripheral blood mononuclear cells stimulated with *M.tuberculosis* antigen and the cells were used for determination of intracellular chemokine positive cells. Chemokine gene polymorphisms are being studied from the DNA extracted from the white cells of the patients and healthy controls. Chemokine gene polymorphisms will be correlated with the level of chemokines as well as intracellular chemokine positive cells. So far, 50 PTB patients and 50 healthy volunteers are studied. Analysis will be carried out after collecting 60-70 subjects in each group.

The study is in progress.

(x) Effect of vitamin D_3 on neutrophil Cathelicidin, Defensin-1α and TLR gene expression in PTB

(Principal Investigator: Dr.P. Selvaraj)

Background: Neutrophils are essential components of the human innate immune system and associated with the first line defense mechanism against

invading microorganisms. Vitamin D_3, a potential immunomodulator, is known to influence innate and adaptive immunity. In the present study, the effect of vitamin D_3 on the innate immune functions of neutrophils in PTB is explored at the molecular level using real-time PCR.

Aim: To find out the vitamin D_3 effect on cathelicidin, defensin-1α and TLR gene expression in neutrophils of PTB patients

Methods: The study was carried out in 20 PTB patients and 20 healthy control subjects. Neutrophils isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation followed by sedimentation in 3% Dextran. Neutrophils were cultured for 18hrs with live *M.tuberculosis* and its CFA in the presence and absence of vitamin D_3. The total RNA extracted was used for cDNA synthesis. The relative quantification for the target genes cathelicidin, defensin-1α, vitamin D receptor (VDR), Cyp27B1, TLR-2, 4, 8, 9 and TIRAP and house keeping gene, β-actin was done using RT-PCR with TaqMan assay primers and probes. The data is being analyzed.

(xi) Effect of vitamin D_3 on intracellular expression of perforin, granulysin and regulatory T-cells in PTB

(Principal Investigator: Dr.P. Selvaraj)

Background: Protective immunity in TB is dependent on the co-ordinated release of cytolytic effector molecules from effector T-cells and the subsequent granule-associated killing of infected target cells. Vitamin D_3 is a potent modulator of macrophage and lymphocyte functions and enhances the exocytosis of cytolytic granules like perforin and granzymes and antimicrobial molecules such as granulysin from CTL. T-regulatory (Treg) cells have been shown to suppress antimicrobial immune responses against intracellular pathogens and protect the host by preventing collateral damage from excessive inflammation. The present study was aimed to understand the effect of vitamin D_3 on regulatory T-cells and intracellular expression of various cytolytic molecules in PTB.

Aim: To find out the effect of vitamin D_3 on intracellular expression of perforin, granulysin and regulatory T-cells in PTB

Methods: The study is carried out in 20 PTB patients and 20 healthy control subjects. Peripheral blood mononuclear cells are cultured for 72 hrs with live *M. tuberculosis* and its CFA in the presence and absence of vitamin $D_{3.}$ After 72 hrs, the cells were processed for immunostaining of CD4, CD8, CD25 and CD56 cell surface markers and intracellular perforin, granulysin and $Foxp3^{+}$ regulatory T-cells by using specific monoclonal antibodies and analyzed in flow cytometry.
The data is being analyzed.

Department of Statistics

Completed studies:

Stochastic model of the growth of HIV in an infected individual

(Principal Investigator: Dr.P. Venkatesan)

Humans are constant victims of infectious diseases. These diseases pose a complex and global threat to human populations. The recent pandemic of the HIV has produced global concern and a greater awareness of infectious diseases in the world. About 5 million people worldwide have died from AIDS so far, with an estimated 33.3 million infected now. The human immune system consists of about 1000 billion cells. Certain immune cells, named T-cells, recognize foreign structures on the surface of other cells. They release substances that alert other immune cells or inhibit growth of infectious agents, or they kill virus infected cells. Other immune cells, named B cells, release antibodies that bind to infectious agents and mark them for elimination. Macrophages are immune cells that digest bacteria or virus-infected cells. Last 50 years have witnessed tremendous advances in our understanding of the molecular biology of the immune system and how it fights against infections. Yet, many important processes are barely understood. For example, in the context of virus infections, there are crucial questions. What is the lifetime of a virus-infected cell in a patient? What proportions of infected cells are eliminated by the immune response before they can release new virus particles? How many virus particles are neutralized by antibodies? What governs the complex steady state among virus, uninfected cells and various components of the antiviral immune response in persistent infections? How does the virus evolve during individual infections after diseases development?

These questions have one common theme: they are of quantitative nature and they deal with the dynamics of populations of immune cells and infectious agents. In order to answer these questions, it is not sufficient to know what detailed molecular interactions exist between individual cells or molecules. One

requires an understanding of the complex dynamics of infectious diseases based on precise stochastic models. This paper, discusses how stochastic models, can improve our understanding of infectious diseases. In addition, the increasing understanding of such biological systems also justifies development of more complex stochastic models designed to provide more detailed descriptions of the dynamics.

Viruses infect cells; infected cells produce viruses. HIV grows predominantly in certain white blood cells. The virus enters the cell by means of a specific interaction between the viral envelope protein and proteins on the surface of the cell. Virus and cell membranes fuse and inner structure of the virus containing the virus genome enters the cell. Following cell entry, the virus RNA genome is transcribed into DNA, which subsequently is inserted into the genome of the host cell. Using the genetic machinery of the host cell, the viral genome starts to generate virus proteins and new RNA copies of itself. Viral proteins and RNA combine to give rise to new virus particles, which are released from the cell. New virus particles then find new target cells and the process is repeated.

Stochastic modeling

Modelling the interaction between HIV-1 virus and CD4 cells has been a major area of research for many years. Mathematical models have come to play an important part in biological system. Mathematics makes it possible to make predictions about the behaviour of the system. Studies by many authors have shown that there is a correlation between the antigen receptors of T-cells and HIV replication. Consequently, the pathogenesis of the infection can be understood only when the genetic variation in HIV and the receptor-specific HIV infection are given their due importance in the formulation of any model of the dynamics of HIV in an infected individual.

Before describing model, we briefly outline the life-cycle of HIV and the events that occur between the time of an infection of HIV with T4 cell and the lysis of the host cell. The HIV is a retrovirus and its RNA carries the genetic information. The HIV has a dense cylindrical core encasing two molecules of the viral genome. Virus-encoded enzymes required for efficient multiplication, such as reverse

transcriptase and integrase, are also incorporated into the viral particle. After attaching itself to the cell wall of the host T4 cell, the virus injects it RNA together with the enzymes reverse transcriptase and integrase into the cytoplasm of the host cell. The viral reverse transcriptase enzyme first synthesizes a single complementary, negative-sense DNA copy to HIV RNA; next the RNA is denatures; and then a complementary positive-sense DNA copy is synthesized to create double-standard proviral DNA.

The proviral DNA may either reside in episomal form or enter the cell nucleus and become integrated into host DNA under the action of the viral integrase enzyme. Within the cell, the proviral DNA (also called Provirus) can remain latent, giving no sign of its presence of several months or years. In this stage, every time the infected cell divides, the provirus is duplicated with the cell's DNA. On the other hand, once the cell activation occurs due to antigen or mitogen, the proviral DNA transcribes viral genomic RNA and messenger RNA (mRNA). The messenger RNA translates the regulator proteins tat and rev. Tat protein promotes transcription of more messenger RNA. Rev protein causes multiple spliced segments of messenger RNA to form singly sliced segments that are translated into structural proteins, envelop proteins and viral enzymes. The assembly of proteins and enzymes, together with the viral genomic RNA are assembled to form mature HIV virus which buds on the cell wall. The ongoing process of building of mature virons on the cell wall take place until the infected cell is unable to withstand the burden of the viral production when the cell undergoes the lysis releasing the mature virons ready to attack other T4 cells.

The replication process has limited efficiency as incomplete, RNA – deficient and damaged virions may be released from the host cell, and viral proteins may be produced in excess during the life-cycle and can be detected while the host cell undergoes lysis. The population of defective virons may inhibit the production of fully mature virons. Accordingly, we proceed to formulate a stochastic model of viral production in a host cell by taking into consideration the fact that along with fully mature HIV virons, damaged virons are also produced at the time of lysis.

We assume that at time t = 0, a HIV attaches to the cell wall of a T4 cell and injects its RNA instantaneously into the cytoplasm of the host cell. Let T be the time at which the viral DNA gets integrated with the host DNA. We assume that viral RNAs are replicated according to a Poisson process with rate λ, $\lambda > 0$. Let N(t) be the number of the viral RNAs that are present inside the cell at time t. we assume that at any time t, the budding of HIV takes places with a rate proportional to N(t). Let X(t) be the number of HIV buds that are present on the cell wall at time t. then the vector process (X(t), N(t)) is Markov and its structure is analyzed in the following section. For brevity, we denote Z(t) = (X(t), N(t)).

The probability generating function of the vector process (X(t), N(t)) defined by

$$G(u, v; t) = E[u^{X(t)} u^{N(t)}].$$

Using Laplace transform method and solving a system of equations we get

$$E[N(t)] = \frac{\lambda}{\mu}\left\{1 - \frac{\mu e^{-\alpha t} - \alpha e^{-\mu t}}{(\mu - \alpha)}\right\};$$

$$E[X(t)] = \lambda t - \frac{\lambda}{\mu - \alpha}\left\{\frac{\mu}{\alpha(1 - e^{-\alpha t})} - \frac{\alpha}{\mu(1 - e^{-\mu t})}\right\};$$

$$E[X(t)N(t)] = \frac{\lambda}{\mu}t - \frac{2\lambda^2\mu}{\alpha(\mu + \alpha)(\mu + 2\mu)(1 - e^{-\alpha t})}$$
$$+ \frac{2\lambda^2\alpha}{\mu^2(\mu + \alpha)}(1 - e^{-\mu t}) + \frac{\lambda^2\alpha}{2\mu^2(2\mu + \alpha)}(1 - e^{-2\mu t});$$

$$E[X(t)\{N(t) - 1\}] = \frac{\lambda^2}{2\mu^2}\left\{1 - \frac{2\lambda^2}{(\mu - \alpha)(2\mu + \alpha)}e^{-\alpha t} + \frac{2\alpha}{\mu - \alpha}e^{-\mu t} - \frac{\alpha}{2\mu - \alpha}e^{-2\mu t}\right\}$$

$$E[X(t)\{X(t) - 1\}] = \lambda^2 t^2 - \frac{4\lambda^2\mu^2}{\alpha(\mu - \alpha)(2\mu + \alpha)}t + \frac{4\lambda^2\alpha}{\mu(\mu - \alpha)}t - \frac{\lambda^2\alpha}{\mu(2\mu - \alpha)}t$$
$$+ \frac{4\lambda^2\mu^2}{\alpha^2(\mu - \alpha)(2\mu + \alpha)}(1 - e^{-\alpha t}) - \frac{4\lambda^2\alpha}{\mu^2(\mu - \alpha)}(1 - e^{-\mu t}) + \frac{\lambda^2\alpha}{2\mu(2\mu - \alpha)}(1 - e^{-2\mu t})$$

Using the above expressions, we can obtain explicitly the correlation coefficient ρ between X(t) and N(t) as

$$\rho = \frac{E[X(t)N(t)] - E[X(t)]E[N(t)]}{\sqrt{EX^2(t) - E[X(t)]^2}\sqrt{EN^2(t) - E[N(t)]^2}}$$

A numerical illustration: For the purpose of illustration we assume α = 200.0, λ = 300.0, μ = 400.0 and obtain the first two moments of X(t) and N(t), the ratio between their means and the correlation coefficient (ρ) between them. The results are highlighted in Tables-1 to 3. Since α = 200, the mean time for the viral RNA to get integrated and start releasing the HIV buds is 0.02. Hence for increasing values t > 0.02 both E[X(t)] and E[N(t)] can be expected to increase. Table-11 shows this trend. We also observed that the released viral RNAs rapidly become buds, the number of buds will increase and the number of viral RNAs will increase which is indicated as negative correlation between X(t) and N(t) in Table-11. At μ increases, E[X(t)] increased but E[N(t)] decreased and hence the ratio between E[X(t)] and E[N(t)] increased (Table-12) and the correlation between X(t) and N(t) remained negative (Table-12). As the rate of releasing viral RNAs increases, both the mean number of buds and the viral RNAs should increase. However, since the rate of buds is a constant, we find that the ratio remains a constant even though the value of λ increases (Table-13). In this case also correlation between X(t) and N(t) remains negative (Table-13).

Conclusion: In the model described above, the dynamics of the growth of HIV inside an infected cell is analysed by a multiplication process. A numerical illustration that brings out the impact of the genetic diversity in viral production also presented. The mean number of HIV buds X(t) that are present on the cell wall at time t the number of viral RNAs N(t) that are present inside the cell at time t have been obtained. As time goes on increasing in Table 11 and for fixed time with μ increases in Table 12, the correlation also increases. However in Table 13 fixed time with increasing λ, the correlation decreases. Contribution of the stochastic models to statistical work is the ability to obtain the covariance structure which is very difficult to obtain in any other model.

Table 11: ρ versus t for α = 200.0, λ = 300.0, μ = 400.0

t	E[X(t)]	E[N(t)]	E[X(t)]/E[N(t)]	P
0.05	12.75014	0.74993	17.00173	-0.09402
0.06	15.75002	0.74999	21.00028	-0.09225
0.07	18.75000	0.75000	25.00004	-0.09064
0.08	21.75000	0.75000	29.00001	-0.08920
0.09	24.75000	0.75000	33.00000	-0.08786
0.10	27.75000	0.75000	37.00000	-0.08661
0.11	30.75000	0.75000	41.00000	-0.08541
0.12	33.75000	0.75000	45.00000	-0.08426
0.13	36.75000	0.75000	49.00000	-0.08316
0.14	39.75000	0.75000	53.00000	-0.08211
0.15	42.75000	0.75000	57.00000	-0.08109

Table 12: ρ versus μ for α = 200.0, λ = 300.0, t = 0.05

μ	E[X(t)]	E[N(t)]	E[X(t)]/E[N(t)]	ρ
400.00	12.75014	0.74993	17.00173	-0.06195
410.00	12.76843	0.73164	17.45173	-0.06059
420.00	12.78584	0.71422	17.90173	-0.05928
430.00	12.80245	0.69762	18.35174	-0.05802
440.00	12.81831	0.68176	18.80175	-0.05679
450.00	12.83346	0.66661	19.25176	-0.05561
460.00	12.84795	0.65212	19.70177	-0.05446
470.00	12.86182	0.63825	20.15178	-0.05335
480.00	12.87512	0.62495	20.60179	-0.05227
490.00	12.88787	0.61220	21.05180	-0.05122
500.00	12.90011	0.59995	21.50182	-0.05020
510.00	12.91188	0.58819	21.95183	-0.04922
520.00	12.92319	0.57688	22.40184	-0.04825
530.00	12.93407	0.56600	22.85186	-0.04732
540.00	12.94455	0.55552	23.30187	-0.04641
550.00	12.95465	0.54542	23.75189	-0.04552
560.00	12.96439	0.53568	24.20191	-0.04466
570.00	12.97379	0.52628	24.65192	-0.04382
580.00	12.98286	0.51721	25.10194	-0.04301
590.00	12.99163	0.50844	25.55196	-0.04221
600.00	13.00010	0.49997	26.00198	-0.04143

Table 13: ρ versus λ for α = 200.0, μ = 400.0, t = 0.05

Λ	E[X(t)]	E[N(t)]	E[X(t)]/E[N(t)]	ρ
300.00	12.75014	0.74993	17.00173	-0.06195
310.00	13.17514	0.77493	17.00173	-0.06392
320.00	13.60015	0.79993	17.00173	-0.06591
330.00	14.02515	0.82493	17.00173	-0.06793
340.00	14.45015	0.84992	17.00173	-0.06997
350.00	14.87516	0.87492	17.00173	-0.07205
360.00	15.30016	0.89992	17.00173	-0.07417
370.00	15.72517	0.92492	17.00173	-0.07633
380.00	16.15017	0.94991	17.00173	-0.07853
390.00	16.57518	0.97491	17.00173	-0.08077
400.00	17.00018	0.99991	17.00173	-0.08307
410.00	17.42519	1.02491	17.00173	-0.08542
420.00	17.85019	1.04990	17.00173	-0.08782
430.00	18.27520	1.07490	17.00173	-0.09030
440.00	18.70020	1.09990	17.00173	-0.09283
450.00	19.12520	1.12490	17.00173	-0.09545
460.00	19.55021	1.14990	17.00173	-0.09814
470.00	19.97521	1.17489	17.00173	-0.10092
480.00	20.40022	1.19989	17.00173	-0.10380
490.00	20.82522	1.22489	17.00173	-0.10677
500.00	21.25023	1.24989	17.00173	-0.10986

Studies in progress:

Proximal support vector machine for disease classification

(Principal Investigator: Dr.P. Venkatesan)

In real world there are problems which cannot be solved using mathematical model or by classical programming techniques. In these areas, machine learning approach plays a vital role. As a broad subfield of artificial intelligence, **machine learning** is concerned with the design and development of algorithms and techniques that allow computers to learn.

Support Vector Machines (SVM) are one of the recently developed machine learning algorithm under the supervised learning approach, from the statistical learning theory implementing the structural risk minimization (SRM) principle. It has been successful in many real world classification problems like handwritten recognition, object recognition, text categorization, image recognition, classification of gene expression and many more.

Unlike neural networks, SVMs minimize the estimation error, keeping the training error fixed. There are many variants of SVM since its existence in the literature. Proximal support vector machines (PSVM) was introduced recently as a variant of SVM for binary classifications. Theoretically, both PSVM and SVM target the optimal Bayes rule asymptomatically, which explains their comparable performance in most studies. However, the PSVM solves a system of linear equations using an extremely fast and simple algorithm and thus demands much less computational effort than the SVM. SVMs are basically binary classifiers but can be extended to multi classification task using either one against one or one against all approaches.

Let us consider the training set D to be $\{(x_i, y_i)\}_{i=1}^{N}, x_i \in R^m$ and the output label $y_i \in \{1,-1\}$, which is separable into two classes. Let w denote the normal vector to the separating hyperplane and $|b|/\|w\|$ is the perpendicular distance from the origin. Thus the hyperplane is given by

$$w \cdot x + b = 0$$

In other words, the basic problem becomes predicting the pair w and b such that the hyperplane is optimal (ie) with maximal margin on either sides of it.

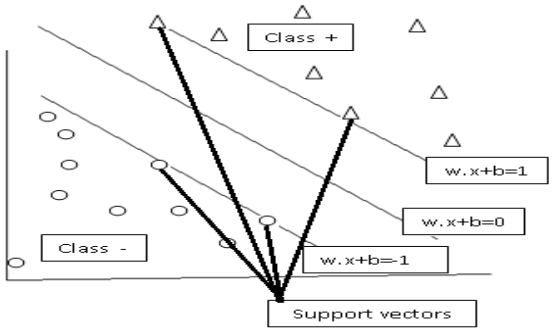
Since the training set is linearly separable, if $y_i = 1$ then $w \cdot x_i + b \geq 1$ and if $y_i = -1$ then $w \cdot x_i + b \leq -1$ for all $x_i \in D$. Combining both $y_i(w \cdot x_i + b) \geq 1 \quad \forall i$

The boundary of each class can be given as for class +, $w \cdot x + b = 1$ for class - , $w \cdot x + b = -1$. The training points that lie on these hyperplanes are called support vectors (Fig. 36). We wish to find the hyperplane such that we can separate the point x_i, according to the function

$$f(x_i) = sign(w \cdot .x_i + b) = \begin{cases} 1, if\ y_i = 1, \\ -1, if\ y_i = -1 \end{cases}$$

Hence the SVM algorithm tries to find the optimal separating hyperplane with the maximum margin.

Fig. 36: Linearly separable data with support vectors



If D is not separable, we shall use the soft margin optimization. The important and inherent constraint in linear separable case is that it assumes that there are no training errors, which is impossible in many of the real world problems. To address this issue, the slack variables $\xi_i's$ are introduced to allow the slight violation of the margin constraint to manage the noisy data $y_i(w \cdot x_i + b) \geq 1 - \xi_i \quad \forall i$

Thus it can be stated as

$$\text{Min } \frac{1}{2} \| w \|^2 + C \sum_{i=1}^{N} \xi_i$$

$$\text{Subject to } \begin{matrix} y_i(w \cdot x_i + b) \geq 1 - \xi_i \\ \xi_i \geq 0 \qquad\qquad \forall i \end{matrix}$$

where C is constant, which is a free parameter and balances the margin maximization and classification violation. In this way it can be regarded as a regularization parameter. It is fixed by the user. Let $\alpha_i's$ be the Larangian multipliers for $y_i(w\cdot x_i+b)-1+\xi_i\geq 0$ and $\mu_i's$ be the Larangian multipliers for $\xi_i\geq 0$. The Larangian primal objective function is

$$\text{Min } L_P=\frac{1}{2}\|w\|^2+C\sum_{i=1}^{N}\xi_i-\sum_{i=1}^{N}\alpha_i[y_i(w\cdot x_i+b)-1+\xi_i]-\sum_{i=1}^{N}\mu_i\xi_i$$

The dual of this is

Maximize $L_D=\sum_{i=1}^{N}\alpha_i-\frac{1}{2}\sum_{i=1}^{N}\sum_{j=1}^{N}\alpha_i\alpha_j y_i y_j x_i\cdot x_j$ subject

to $0\leq\alpha_i\leq C \quad and \quad \sum_{i=1}^{N}\alpha_i y_i=0$.

The advantage of the concept of the slack variables $\xi_i's$ is that $\xi_i's$ does not appear in the dual formulation. Therefore, in this case also the solution becomes $w=\sum_{i=1}^{N_s}\alpha_i y_i x_i$ where N_S is the number of support vectors, whereas the bias b can be found using Karush Kuhn Tucker conditions for the primal. Therefore optimal hyperplane $w\cdot x+b=0$ has been constructed. The decision function is $f(x)=sign(w\cdot .x+b)=sign(\sum_{i=1}^{N_S}\alpha_i y_i x_i\cdot x+b)$

In case of nonlinear separable data, the SVM first maps the input to high dimensional feature space wherein the data are separated. This mapping is done through the functions called kernels. Let $z=\phi(x)$ be the function defined on the input x such that $z_i\cdot z_j=\phi(x_i)\cdot\phi(x_j)=K(x_i,x_j)$

from R^m to the feature space Z, where K is the kernel satisfies the Mercer's conditions. Now the idea is to find the hyperplane $w\cdot z+b=0$ so that we can separate the point in the feature space with this hyperplane. Incorporating (14), the nonlinear separating hyperplane is the solution of

Maximize $L_D = \sum_{i=1}^{N} \alpha_i - \frac{1}{2} \sum_{i=1}^{N} \sum_{j=1}^{N} \alpha_i \alpha_j y_i y_j K(x_i \cdot x_j)$ subject to $0 \le \alpha_i \le C \quad and \quad \sum_{i=1}^{N} \alpha_i y_i = 0$. And the decision function is

$$f(x) = sign(w \cdot z + b) = sign[\sum_{i=1}^{N_S} \alpha_i y_i K(x_i \cdot x) + b]$$

The standard SVM problem can be considered as classifying m points into two classes in the n-dimensional real space R^n, represented by the mxn matrix A. Let D be mxm diagonal matrix with +1 or -1 along its diagonal according to the class of the corresponding to the data instance.

These modifications change the nature of the optimization problem significantly, as the explicit exact solution can be given as $w = A'Du; \ \gamma = -e'Du; \ \ y = \frac{u}{\vartheta}$ with $u = \left(\frac{1}{\vartheta} + HH' \right)^{-1} e$ where H = *D*[*A*- *e*] using Larangian formulation and KKT optimality conditions. A similar theory of the nonlinear version can be done. The only change in the final solution is that matrix A is replaced by the kernel matrix.

Application to spinal TB data

The data consisted of 108 patients admitted to a randomized clinical trial. The patients had TB involving the thoracic or lumbar spine, allocated randomly to one of the three treatment series. The three treatment series were:

Rad 6: Modified 'Hong Kong' operation of radical resection of the lesion and insertion of autologus bone grafts. In addition, patients had INH plus RMP for 6 months.

Amb 6: Ambulant from the start of chemotherapy and received INH plus RMP for 6 months.

Amb 9: Ambulant from the start of chemotherapy and received INH plus RMP for 9 months.

The variables considered were Age, Gender (M-1, F-0), treatment (Rad-1, Amb6-2, Amb9-3) Fusion of bones in months, the angle of kyposis, Site of disease

(Thoracic -1, Thoracic lumbar-2, Lumbar-3), Number of vertebrae involved, Total vertebral body loss, Sinuses and abscesses. The objective was to classify whether patients had favorable response or not and whether the disease was radiographically quiescent or not. We used PSVM Matlab code and libSVM code to build the model for this classification. Matlab 7.5 was used for both the models. The parameter selection for nonlinear PSVM was done through uniform designs tables. The results are given below in the following table (Table 14).

Table 14: Classification rates using PSVM & SVM

Formulation		Classification rates(%)	
		Training	Testing
PSVM	Linear	89.09	87.82
	nonlinear	90.64	86.18
SVM	Linear	100	91.18
	nonlinear	100	85.29

The results shwoed that linear SVM had better accuracy of classification rates in the testing set, whereas the PSVM had approximately 87% in both cases. The classification rates were obtained with 10-fold cross validation as performance measure. However, the time to train the model was much lesser in the case of PSVM. This approach of diagnosing the disease with the selection of parameters through uniform designs table was quite promising one.

Biomedical Informatics

Studies completed:

(i) PE and PPE proteins of *M. tuberculosis* as potential antigens

(Principal Investigator: Dr. Luke Elizabeth Hanna)

The emergence of drug resistant strains of *M. tuberculosis* (MTB) and co-infection with HIV makes TB a more serious public health problem than ever, emphasizing the need for a novel vaccine for TB. About 10% of the MTB genome code for two large unrelated families of acidic, glycine-rich proteins, namely, PE and PPE families. The PE and PPE genes are reported not to be present in any non-mycobacterial species, or in closely related bacteria such as *Nocardia farcinica*, and therefore constitute a unique class of proteins. Several PE-PGRS proteins are localized on the extracellular surface of MTB and thus, like other cell wall constituents, are able to actively traffic out of the phagolysosome and gain access to both the intracellular compartments of the infected host cells, as well as to the extracellular environment. Therefore, PE/PPE proteins deserve attention and evaluation for their potential as antigens that can comprise a TB vaccine. We undertook an effort to prioritize these proteins based on the relevance to latency. PE-PGRS/PPE proteins of H37Rv associated with dormancy were identified from the MTBreg database and promiscuous CTL epitopes were predicted from a set of high priority proteins using two different *in silico* methods. The possible population coverage of the epitopes shortlisted by both methods was calculated using the population coverage calculator available in IEDB analysis resource.

We found that nearly half of the PE/PPE family proteins were found to be essential for dormancy of MTB. Three proteins of this family (Rv0872c, Rv1168c and Rv1169c) known to be upregulated under multiple dormancy associated conditions were chosen for further characterization. Thirteen promiscuous epitopes were identified in these proteins. These epitopes were found to be conserved in 7 different virulent strains of MTB and had a population coverage of

71 - 94%. The current study demonstrated that PE/PPE proteins that are important for dormancy in MTB also encode a number of promiscuous epitopes that could form important components of a successful TB vaccine. This study could be followed up by a large study to identify promiscuous epitopes from all PE/PPE proteins and pick the best of these candidates for vaccine formulation. The study is completed.

Studies in progress:

(i) Identification of physico-chemical properties that characterize known epitopes and non-epitopes

(Principal Investigator: Dr. Luke Elizabeth Hanna)

About 15 million (>25%) of the 57 million annual deaths worldwide are due to infectious disease. Infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, parasites or fungi; the diseases can be spread directly or indirectly, from one person to another. The best possible way to eradicate or to control most infectious diseases is through vaccination. Recent developments in bioinformatics have paved the way for Reverse Vaccinology. Though various methods are available to predict immunodominant epitopes, it is reported that there is no perfect correlation between the *in silico* prediction and *in vitro* immune response. Hence, there is need to improve the existing methods for prediction of immunodominant epitopes for vaccine discovery. It is important to understand the underlying salient features of epitopes that distinguish them from non-epitopes. Hence, we undertook a large scale bioinformatics analysis to identify salient physico-chemical characteristics of epitopes that differentiate them from non-epitopes. More than 1000 known epitopes and an equal number of reported non-epitopes were obtained from IEDB and analyzed for 24 different physical and chemical properties. The study is on going.

(ii) Molecular modeling of *M. tuberculosis* proteome

In spite of advances in technology, experimentally solved structures (by X-RAY/NMR) are available only for 328 proteins of MTB, which covers less than 10 percentage of the total MTB genome. Understanding structures of proteins will

not only yield valuable clues to their function, but will also be useful for motif finding, protein docking and off-target identification. The present study is an attempt to predict three dimensional structures for MTB proteins for which experimental structures are not available. Till date, 125 proteins have been analyzed. The study is ongoing.

Epidemiology

Completed study:

(i) Sample survey to estimate tobacco use in urban, semi-urban and rural areas from Tamil Nadu

(Principal Investigator: Dr.C. Kolappan)

Tobacco use is considered to be one of the preventable causes of death in the world. In this survey, data on tobacco use from individuals 15 years and above through a community-based sample survey in three groups namely urban, semi-urban and rural population in Tamil Nadu was collected. The required sample size was calculated as 2141 per group.

The distribution of the study participants according to the study area was as follows:

- Urban (Chennai) - No. of Wards: 31; adult population ≥ 15 years who were enumerated in each ward was 90 (Slum 30 and Non Slum 60)
- Semi Urban (Ambattur) - No. of Wards: 10; adult population ≥ 15 years who were enumerated in each ward was 240 (Slum 80 and Non Slum 160)
- Rural (Sriperumbuthur) - No. of villages: 31; adult population ≥ 15 years who were enumerated in each village was 90 (colony 30 and main village 60).

The survey was conducted from August, 2009 to September, 2011. A written consent was obtained from all the participants and the registered population was screened for PTB and tobacco use using a structured questionnaire; two sputum specimens were collected from all the study participants. The overall coverage is shown in table 15.

Table 15: Coverage of survey participants

	No. Wards / villages	No. to be screened	No. actually screened	Covarage (%)
Rural	31	2790	2608	93
Semi Urban	10	2400	2254	94
Urban	31	2790	2648	95
TOTAL		7980	7510	94

It was found that the use of tobacco was very negligible among women. Hence, further analysis of the prevalence of TB based on tobacco usage was restricted only to men. The prevalence of tobacco usage was analysed in two broad categories: smoke (cigarette, beedi, pipe and cigar) and smokeless tobacco use (chewing, snuff, pan masala and others) (Table 16). Of the 7510 individuals screened, 1604 (21%) were tobacco users. Sputum samples were collected for 6639 (88%) of the individuals.

Table 16: Prevalence of TB among different categories of tobacco users

Area	**Tobacco usage category**	**No. Screened**	**Sputum collected**	**Smear or culture positive**	**Crude TB prevalence (per 1000 population)**
	Smoke	312	278	4	**14**
	Smokeless	246	219	1	5
Rural	Both	61	54	1	19
	None	1989	1850	6	3
	Total	2608	2401	12	**5**
	Smoke	254	228	2	**9**
	Smokeless	158	142	0	0
Semi Urban	Both	60	55	0	0
	None	1782	1595	6	4
	Total	2254	2020	8	**4**
	Smoke	277	241	0	0
	Smokeless	185	151	2	13
Urban	Both	51	44	0	0
	None	2135	1782	13	7
	Total	2648	2218	15	**7**
Overall		7510	6639	35	**5**

The survey has been completed and detailed data analysis is in progress.

Studies in progress:

(i) Estimation of prevalence of PTB in Chennai city

(Principal Investigator: Dr.C. Kolappan)

Since the prevalence of PTB is high in urban areas, this sample survey was started in the Chennai metropolitan. The first survey was conducted between July, 2010 and October, 2011. Assuming a prevalence of 400/100000 population, 25% precision design effect of 1.3 and 25% loss to coverage, the sample size was estimated to be 26,529. The sample size was distributed among 50 clusters with a cluster size of 531. An adult population of 600 individuals (($\geq$15 years of age) were enumerated in each ward (Slum - 200, Non Slum - 400). A sample size of 30,000 (600 population in 50 wards) is screened for TB by chest symptoms and chest radiography using MMR (mass miniature radiograph) as shown in the following table 17.

Table 17: Details of population screening

Activities	Coverage
Enumeration	29998
Symptom screening	27800 (93%)
X-ray screening	27156 (91%)
Sputum eligible	2749
Sputum collection	2508 (91%)
Smear and/or culture positives	95

Culture and drug susceptibility testing are in progress.

The second survey is being conducted since October 2011 to measure the change in prevalence of TB in the Chennai metropolitan. The coverage upto March 2012 is shown in the following table 18.

Table 18: Details of population coverage

Activities	Coverage
Enumeration	11440*
Symptom screening	10671 (93%)
X-ray screening	7193
Sputum eligible	1254
Sputum collection	1127 (90%)
Smear and/or culture positives	31

* Only Symptom screening in first 6 wards.

The survey is in progress.

Electronic Data Processing

The major role of the Electronic Data Processing division is to provide the data entry/verification support for the research in TB studies conducted in clinic, epidemiology, laboratory, operational research and TB control program activities. Also, this division supports for data management, prepares pre-printed forms and reports for field activity of large scale epidemiological surveys, and generates data tabulations, data analysis and helps in publication of research work.

Information processing, information sharing within the institute, accessing scientific journals and e-mailing are the key requirements for our research organization. The existing IT equipments are being maintained under comprehensive annual maintenance contract. This includes managing the installation of the IT related facilities and ensuring that the IT equipments are maintained and kept up to-date.

The Local Area Networking (LAN) and the video conferencing facilities are well utilized by the researchers, students and trainees. The management of functionality of LAN facility is carried out with the support given by NIH-ICER project. The video conferencing facilities are maintained by project staff attached to ICMR-HCL project and NIH-ICER project. Also the video conferencing facility is established to connect between NIRT, Chennai and Madurai unit of NIRT.

The quantum of documents of epidemiological, clinical, laboratory and program based studies entered and verified from April, 2011 to March, 2012 is shown below (Table 19).

Table 19: No. of documents computerized

SL.NO.	STUDY	NO. OF DOCUMENTS ENTERED	VERIFIED
1	CHENNAI - CLINIC	9577	9436
2	MADURAI - CLINIC	5952	5886
3	CHENNAI DISEASE PREVALENCE - I	41361	41009
4	CHENNAI DISEASE PREVALENCE - II	26399	18378
5	TOBACCO USE	5042	5555
6	FILARIASIS-TB	7618	8527
	TOTAL	95949	88791

A total of 74, 698 records were processed for the Chennai disease prevalence surveys undertaken by epidemiology unit of NIRT. The data analysis was completed for the third repeat disease prevalence survey and a report was written and sent for publication during the period. Data analysis was undertaken for the tuberculin skin test surveys conducted during 1999 to 2010 to study a trend in TB infection.

(Contact person: Mr.R. Subramani)

International Centre of Excellence in Research

Completed studies:

(i) Circulating microbial products and acute phase proteins as markers of pathogenesis in lymphatic filarial disease

(Principal Investigators: Dr. Subash Babu and Dr. V. Kumaraswami)

Background: Lymphatic filariasis can be associated with development of serious pathology in the form of lymphedema, hydrocele, and elephantiasis in a subset of infected patients. Dysregulated host inflammatory responses leading to systemic immune activation are thought to play a central role in filarial disease pathogenesis.

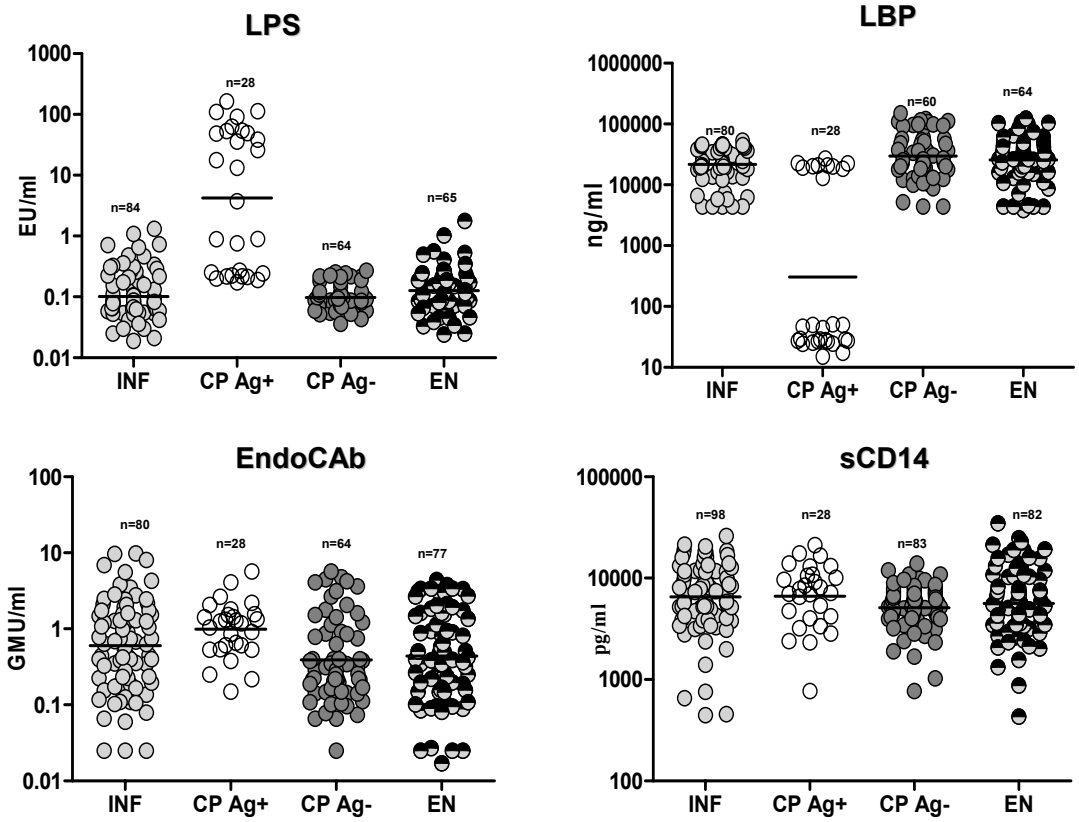
Methods: We measured the plasma levels of microbial translocation markers, acute phase proteins, and inflammatory cytokines in individuals with chronic filarial pathology with (CP Ag+) or without (CP Ag–) active infection; with clinically asymptomatic infections (INF); and in those without infection (endemic normal [EN]) by ELISA and immunoassays. Comparisons between the two actively infected groups (CP Ag+ compared to INF) and those without active infection (CP Ag– compared to EN) were used preliminarily to identify markers of pathogenesis. Thereafter, we tested for group effects among all the four groups using linear models on the log transformed responses of the markers.

Results: We show that circulating levels of LPS (Figs. 37 and 38), acute phase proteins and certain cytokines are significantly elevated in filarial disease with active infection but not in the other groups indicating that filarial infection induced increased production of these factors plays a major role in the development of filarial lymphatic pathology.

Conclusion: Our data suggest that circulating levels of microbial translocation products (lipopolysaccharide and LPS-binding protein), acute phase proteins (haptoglobin and serum amyloid protein-A), and inflammatory cytokines (IL-1β, IL-12, and TNF-α) are associated with pathogenesis of disease in lymphatic filarial infection and implicate an important role for circulating microbial products and

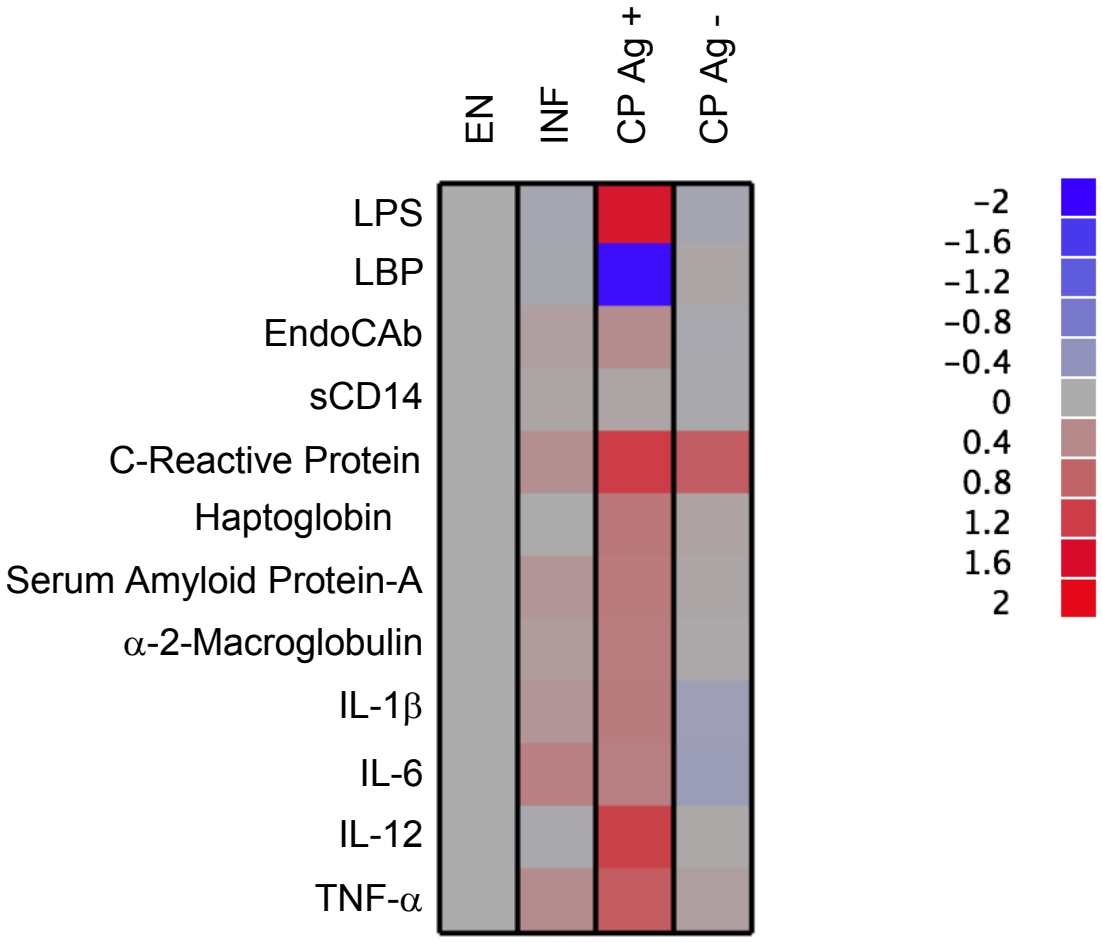
acute phase proteins in establishing the chronic inflammatory milieu in this process.

Fig. 37: Filarial lymphedema is associated with elevated levels ofLPS



Plasma levels of LPS, LBP, EndoCAb and sCD14 from asymptomatic infected [INF] individuals; filarial lymphedema individuals with active infection [CP Ag+]; filarial lymphedema individuals without active infection [CP Ag-] and endemic normal [EN] individuals were measured by ELISA and immunoassays.

Fig. 38: Heatmap depicting circulating microbial products, acute phase proteins and inflammatory cytokines in CP Ag+ individuals compared to EN, INF and CP Ag- individuals



Data (and scale) are log10 geometric mean fold change from EN for each of the analytes measured for each of the groups.

(ii) Altered circulating levels of matrix metalloproteinases and inhibitors associated with elevated type 2 cytokines in lymphatic filarial disease

(Principal Investigators: Dr. Subash Babu and Dr. V Kumaraswami)

Background: Infection with *Wuchereria bancrofti* can cause severe disease characterized by subcutaneous fibrosis and extracellular matrix remodeling. MMPs are a family of enzymes governing extracellular remodeling by regulating cellular homeostasis, inflammation, and tissue reorganization, while tissue-inhibitors of

metalloproteinases (TIMPs) are endogenous regulators of MMPs. Homeostatic as well as inflammation-induced balance between MMPs and TIMPs is considered critical in mediating tissue pathology.

Aim: To elucidate the role of MMPs and TIMPs in filarial pathology

Methods: We compared the plasma levels of a panel of MMPs, TIMPs, other pro-fibrotic factors, and cytokines in individuals with chronic filarial pathology with (CP Ag+) or without (CP Ag–) active infection to those with clinically asymptomatic infections (INF) and in those without infection (endemic normal [EN]) by ELISA and immunoassay. Markers of pathogenesis were delineated based on comparisons between the two actively infected groups (CP Ag+ compared to INF) and those without active infection (CP Ag– compared to EN).

Results: Our data reveal that an increase in circulating levels of MMPs and TIMPs is characteristic of the filarial disease process *per se* and not of active infection; however, filarial disease with active infection is specifically associated with increased ratios of MMP1/TIMP4 and MMP8/TIMP4 as well as with pro-fibrotic cytokines (IL-5, IL-13 and TGF-β).

Conclusion: Our data suggest that while filarial lymphatic disease is characterized by a non-specific increase in plasma MMPs and TIMPs, the balance between MMPs and TIMPs is an important factor in regulating tissue pathology during active infection.

(iii) Expansion of pathogen specific mono – and multi – functional Th1 and Th17 cells in tuberculous lymphadenitis

(Principal Investigators: Dr. Subash Babu, Dr. M.S. Jawahar & Dr. Banurekha)

Background: Th1 and Th17 responses are known to play an important role in immunity to PTB, although little is known about their role in extrapulmonary forms of TB.

Aim: To identify the role of Th1, Th17, and Th22 cells in TB lymphadenitis (TBL)

Methods: We examined mycobacteria–specific immune responses in the whole blood of individuals with PTB (n = 20) and compared them with those with TBL (n = 25). We used multi-parameter flow cytometry with surface phenotyping and

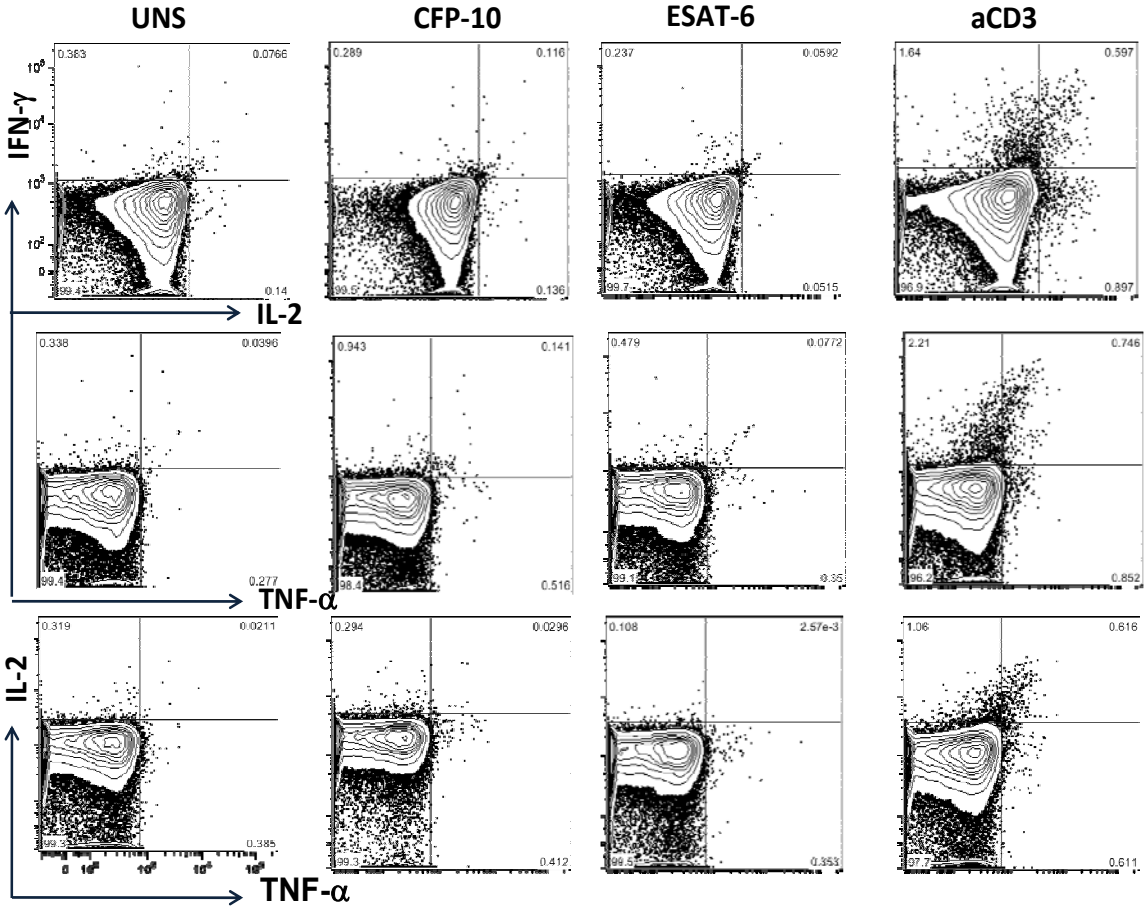
intracellular cytokine staining following stimulation of whole blood with TB antigens as well as anti-CD3 for 6 hours.

Results: Elevated frequencies of $CD4^+$ T-cells expressing IFN-γ, TNF-α, and IL-2 were present in individuals with TBL compared with those with PTB at homeostasis (baseline) as well as in response to mycobacterial antigens ESAT-6 and CFP-10 (Figs. 39 – a, b and c). Similarly, increased frequencies of $CD4^+$ T-cells expressing IL-17A, IL-17F, and IFN-γ were also present in individuals with TBL at baseline and following ESAT-6 and CFP-10 stimulation although no significant difference in the frequency of Th22 cells was observed between the two groups. Finally, frequencies of Th1 (but not Th17) cells exhibited a significantly negative correlation with natural regulatory T-cell frequencies at baseline.

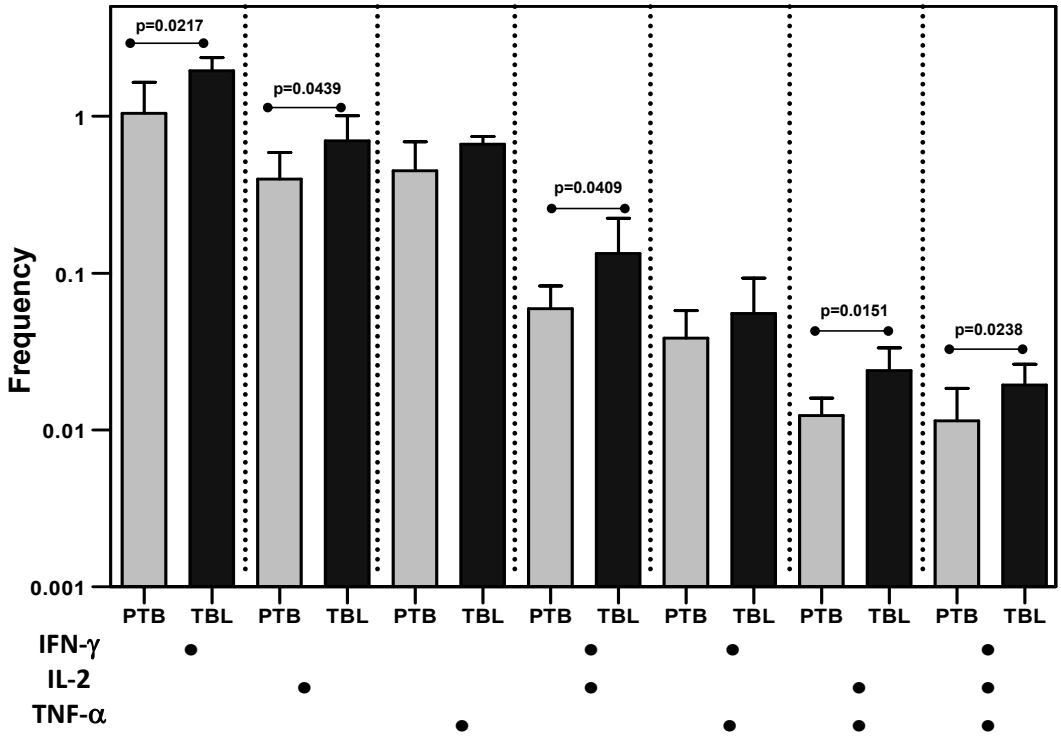
Conclusion: TB lymphadenitis is characterized by elevated frequencies of Th1 and Th17 cells, indicating that Th1 and Th17 responses in TB disease are probably correlates of disease severity rather than of protective immunity.

Fig. 39: Elevated baseline and antigen-specific frequencies of Th1 cells in TBL

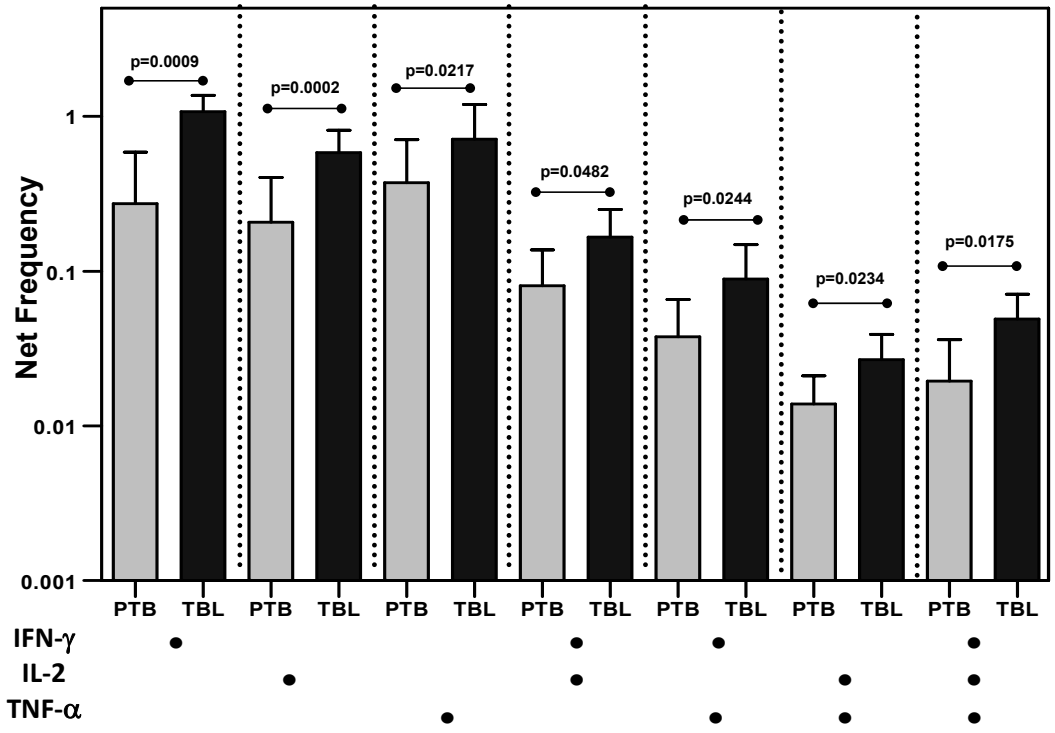
(*A*) Representative whole-blood intracellular cytokine assay flow data from a TBL individual showing expression of IFN-γ, IL-2, and TNF-α. The plots shown are gated on $CD3^+CD4^+$ T cells. (*B*) Baseline frequency of $CD4^+$ T cells expressing one, two, or three cytokines (IFN-γ, IL-2, TNF-α) is shown as bar graphs. The bar represents the geometric mean of the frequency of $CD4^+$ T cells expressing the respective cytokine(s), and the error bar represents the 95% confidence interval in TBL (*n* = 25) and PTB (*n* = 20) individuals. (*C, D*) Net frequency of $CD4^+$ T cells expressing one, two, or three cytokines in response to CFP-10 (*C*) and ESAT-6 (*D*) is shown in TBL and pulmonary TB (PTB) individuals. (*E*) Net frequency of $CD4^+$ T cells expressing the different cytokines in response to anti-CD3 stimulation is shown in TBL and PTB individuals. *P* values were calculated using the Mann-Whitney test.



Baseline



CFP-10



(iv) Immunological profiles of filarial and intestinal helminth infections (Principal Investigators: Dr. Subash Babu, Dr. V Kumaraswami & Dr. C. Kolappan)

Background: Parasitic helminths are complex eukaryotic organisms, characterized by their ability to maintain long-standing, chronic infections in human hosts, sometimes lasting decades. Hence, parasitic helminths are a major health care problem worldwide, infecting more than two billion people, mostly in developing countries. Common helminth infections, such as intestinal helminths, filarial and schistosome infections are a major medical, social and economic burden to the countries in which these infections are endemic. Chemotherapy, while highly successful in some areas, still suffers from the disadvantages of the length of treatment, the logistics involved in the distribution of drugs and in some cases, the emergence of drug resistance. Vector control measures are at best an adjunct measure in the control of helminth infections but also suffer from the same social, logistic and economic obstacles as mass chemotherapy. Therefore, the study of the immune responses to helminth infections attains great importance both in terms of understanding the parasite strategies involved in establishing chronic infection and in the delineation of a successful host immune response to develop protective vaccines against infection.

Objectives: The baseline immunological profile of individuals in an endemic area with filarial and intestinal helminth infections is not well characterized. Our objective was to characterize the ex vivo immune profile as well as the baseline levels of cytokines and other immune parameters in individuals with filarial infections, hookworm infections and other intestinal helminths.

Methodology and Results: We screened 1677 individuals living in villages in Kanchipuram district by ELISA for circulating filarial antigen (which is diagnostic for filarial infection) and stool microscopy for ova and parasites (which is diagnostic for most intestinal helminths). We identified 129 individuals who were filaria positive and 365 individuals who were intestinal helminth positive in this study area. We

obtained blood samples from a subset of these individuals and the immunological characterization is ongoing.

While patient recruitment has been terminated, immunological studies on stored samples are still ongoing.

Studies in progress:

(i) Characterization of immune responses in treatment induced latency in PTB

(Principal Investigators: Dr. S Subash Babu and Dr. M S Jawahar)

The immune responses in latent TB are poorly understood. While it is difficult to define the onset of latency during natural infection, patients undergoing treatment for TB are driven into a state of latency or cure. The present study on the effect of 3 and 4 month regimens containing MFX in sputum smear and culture positive PTB offers us the opportunity to study definitive immune responses pre and post treatment. We are evaluating a variety of innate and adaptive immune responses in patients before and after treatment and our study is comparing the differences in immunophenotype (eg. Markers of T, B and NK cell activation, proliferation and regulatory phenotype) and function (eg. production of cytokines, proliferative responses to TB antigens) at different time points following treatment. The kinetics of immune responses in patients who relapse are used to assess immunological predictors of relapse in TB. In addition, we are also trying to determine immunological differences between PTB, extrapulmonary TB, latent TB and uninfected individuals. We have performed *ex vivo* phenotyping on a variety of T, B, NK, DC and monocyte markers including regulatory T-cells, plasmacytoid and lymphoid dendritic cells, regulatory B cells and inflammatory monocytes on all our samples. We have recruited over 110 patients in this study thus far and recruitment and follow up is ongoing.

National Institute for Research in Tuberculosis (ICMR)

(Formerly Tuberculosis Research Centre)
INSTITUTIONAL ETHICS COMMITTEE

No: 1, Mayor Sathyamoorthy Road, Chetput, Chennai – 600 031, Tamil Nadu, India

Annual Report 2011

National Institute for Research in Tuberculosis Institutional Ethics Committee conducted six meetings (four scheduled and two unscheduled) during the calendar year 2011. All the meetings satisfied the quorum requirements.

The Chair, the Member Secretary, and one member were present during all the 6 meetings; out of the remaining seven members, three members were present for 5 meetings, three members were present for 4 meetings, and one member was present for 2 meetings.

Fifteen new protocols and three re-submissions were reviewed during the year 2011; one new protocol that was registered and approved during the previous years was re-reviewed and approved. Thirty seven ongoing project reviews were also conducted; eight studies have not yet been initiated.

Project Submissions	Tuberculosis		HIV		HIV TB		Filariasis		Vaccine		Total		
	New	Ongoing	New	Ongoing	New	Ongoing	New	Ongoing	New	Ongoing	New	Ongoing	Total
Clinical Trials	2	5		2		3		1		2	**2**	**13**	**15**
Public Health													
Epidemiology	1	2									**1**	**2**	**3**
Programme OR	1	1	1		2						**4**	**1**	**5**
Laboratory													
Bacteriology				1								**1**	**1**
Immunology	1	3	1	2			1		1		**4**	**5**	**9**
Pharmacology			1	2	1	1					**2**	**3**	**5**
Social Sciences	2	2	1	2							**3**	**4**	**7**
Total	**7**	**13**	**4**	**9**	**3**	**4**	**1**	**1**	**1**	**2**	**16**	**29**	**45**

Seven case study files were closed as the projects were completed and summary report submitted.

Out of the 15 new protocols, nine protocols were approved in one submission and three protocols were approved in two submissions. Three protocols were yet to be approved.

Some of the salient features that figured during the year 2010 were:

1. 'Continued Bioethics Education' discussions were continued during the course of the meetings and via electronic mail
2. In continuation with NIRT's US Federal Wide Assurance, an annual report on possible research misconduct for the year 2010 was filled with the Office of Research Integrity (ORI), US Department of Health & Human Services
3. 'Unscheduled' meetings will be held only when there is a compelling reason to do so
4. Name change of the Committee to National Institute for Research in Tuberculosis Institutional Ethics Committee

Selected highlight of ethical concerns:

Over the years, both during initial and continuing review, the Committee had addressed ethical issues regarding conduct of randomized controlled trials for shortening tuberculosis treatment duration, phase I vaccine trials, pediatric studies, and multi centric industry driven research, to name a few

Member Secretary

Chair

Library & Information Centre

The Library plays a key role in expanding the research mission of NIRT. It has sustained the core services and products this year. A range of services such as, access to electronic resources, current awareness service, document delivery service, e-Mail co-ordination, reference assistance, resource sharing and facilitates Internet browsing and digital medial resources are offered. In addition to a collection of over 16, 000 volumes of books and journals, it also holds CD-ROMs, Gratis materials, photographs, reprints, slides, theses, video cassettes and WHO Publications.

Online services: NIRT Digital Library has its homepage and provides web-based full-text access to online journals, online books and databases through its intranet portal. Besides subscribed full-text online resources, it provides gateway to ICMR consortium journals, ICMR resource sharing portal JCCC@ICMR and open access resources.

Information alert services: The library provides alert services to its user group about the latest information on TB like:

- Latest literature on TB
- Latest online Journal (issue) published
- Newly added journals on Open Access Portal
- Forthcoming conference, seminars, symposium, workshops etc.

As part of our Value Added Services, an in-house publication, "**TB Alert**" is being published among ICMR institutes.

(Contact person: Mr.R. Rathinasabapati)

APPENDICES

LIST OF PUBLICATIONS

Publications in Journals : 69

Published	i)	International	: 58
	ii)	National	: 11
Accepted	i)	International	: 36
	ii)	National	: 3

International:

2011:

1. Arun N, Venkatesan P. Recent advancements in causal inference. Int J Pharmaceut Studies Res.2011;II(IV):10-19.

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International:

2012:

1. Aparna JC, Dharman Karthika D, Dhandapaani G, Kannan P, Gupta UD, Gupta P, Ignacimuthu S, Narayanan S. Epitope based recombinant BCG vaccine elicits specific Th1 polarized immune responses in BALB/c mice. Vaccine.2012;30(7) 1364-1370.

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31. Selvakumar N, Syam Sunder A, Gomathi Sekar M, Ponnuraja, Kumar V. Performance indicators of fluorescence microscopy for sputum samples in pulmonary tuberculosis. Int J Mycobacteriology.

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34. Swaminathan S, Banu Rekha VV. Antigen detection as a point-of-care test for TB: the case of lipoarabinomannan. Future Microbiol.

35. Thomas B, Mimiaga MJ, Mayer KH, Perry NS, Swaminathan S, Safren SA. The influence of stigma on HIV risk behavior among men who have sex with men in Chennai, India. AIDS Care.

36. Zumla A, Abubakar I, Raviglione M, Hoelscher M, Ditiu L, McHugh TD, Squire SB, Cox H, Ford N, McNerney R, Marais B, Grobusch M, Lawn SD, Migliori GB, Mwaba P, O'Grady J, Pletschette M, Ramsay A, Chakaya J, Schito M, Swaminathan S, Memish Z, Maeurer M, Atun R. Drug-resistant tuberculosis-current dilemmas, unanswered questions, challenges, and priority needs. J Infect Dis.

National:

1. Anbarasu S, Selvakumar, Kumar V. Newer diagnostic tools in tuberculosis. J Ind Ass Appl Microbiol.

2. Gomathi Sekar M, Rehman F, Kumar V, Selvakumar N. Equivalence of acid alone or acid-alcohol as decolorizing agent in ZN method. Indian J Tuberc.

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Awards/Honours

- Life-time achievement award presented by Indian Oriental Heritage, Kolkata in February 2012 – Dr. Vanaja Kumar

- PCT filing for new antituberculous antibiotic from marine actinomycete strain R2 done on 1st February 2012 – Dr. Vanaja Kumar

- Best poster award for the paper titled, "Diminished type 1 but unaltered type 2 cytokine responses at homeostasis in hookworm infection" at 23rd National Congress of Parasitology held at Anna University, Chennai during November 2011 – Mr. Jovvian George P.

- Best poster award for the paper titled, "Regulation of IL-10 family of cytokines in lymphatic filariasis: a possible role for IL-19 and IL-24 in the prevention of pathology" at 5th Congress of the Federation of Immunlogical Societies of Asia Oceania (FIMSA) held at New Delhi during March 2012 – Ms. Anuradha R.

- Best oral presentation award for the paper titled, **"**Evidence of microbial translocation associated with diminished frequencies of CD8+ T-cells and dendritic cells in hookworm infections" at the Advance Course for Basic and Translational Immunology held at New Delhi during March 2012 - Mr. Jovvian George P.

Workshops/Conferences/Seminars/Training programmes conducted or organized by the Institute.

1. "Workshop on Cochrane Library" on September 16, 2011 at N I R T.

Ph.D. Scholars

List of staff / students who have obtained their Ph.D. degree (Part time/Full time) from the University of Madras

Sl. No.	Name of the candidates	Title of the Ph.D. thesis	Part time / Full time	Supervisor/ Guide
1.	Mr.Madhan Kumar M.	Immune response to Esat-6 & CFP-10 in *M. tb* infection	Full time	Dr. Alamelu Raja
2.	Mr. Azger Dusthackeer V.N.	Luciferase reporter phages for rapid deteciton of dormant & active tubercle bacilli	Part time	Dr. Vanaja Kumar
3.	Mr. Basirudeen S	Role of Interferon gamma release assay in the diagnosis of TB	Full time	Dr. Alamelu Raja
4.	Ms. Aparna J Christy	Development of recombinant BCG based epitope vaccine candidates for TB	Full time	Dr. Sujatha Narayanan
5.	Mr. Balaji S	Bacteriophages & their lysins as alternative to antibiotics for detection of *M. tb* in liquid media	Full time	Dr. Vanaja Kumar

List of ICMR-PDF students:

1. Dr. D. Anbarasu - Project guide Dr. Alamelu Raja.
2. Dr.S. Lakshmi – Project guide Dr. Sujatha Narayanan.
3. Dr. Kaustuv Nayak – Project guide Dr. Sudha Subramanyam.
4. Dr. Jerrine Joseph – Project guide Dr. Vanaja Kumar.

List of staff/students who have submitted their Thesis and waiting for their Ph.D. degree from the University of Madras (Full time and Part time)

Sl.No.	Name of the candidate	Title of the Ph.D. thesis	Part / Full time	Supervisor/Guide
1.	Ms. Neema Boruai	Penicillin binding protein from *M. tuberculosis* & *M. smegmatis*	Full time	Dr. Sujatha Narayanan
2.	Mr. Dinesh Kumar P.	A molecular approach to the role of serine/ threonine kinase PknE in signal transduction involved in host pathogen interactions	Full time	Dr. Sujatha Narayanan
3.	Mr.M. Radhakrishnan	Anti-TB drugs from actinomycetes	Full time	Dr. Vanaja Kumar
4.	Mr. Arunkumar N.	Causal analysis	Part time	Dr.P. Venkatesan

List of students who have registered (full-time) for their Ph.D. programme with the University of Madras

Sl.No.	Name of the Candidate	Source of Funding	Title of the Ph.D. thesis	Supervisor/Guide
1.	Mr. Pugazhvendhan P.	ICMR	Immunoproteomic identification of B-cell antigens of *M. tb*	Dr. Alamelu Raja
2.	Ms.D. Santhi	ICMR-TASK FORCE	Novel subunit vaccine targets from *M. tb*	Dr. Alamelu Raja
3.	Ms.Maddineni Prabhavathi	CSIR	Comparative genomics and pathogenesis of TB	Dr. Alamelu Raja
4.	Mr.S. Balaji	ICMR	Diagnostic evaluation of novel T-cell (Rv2204c, Rv2394) antigens of *M. tb*	Dr. Alamelu Raja
5.	Ms.G. Akilandeswari	INSPIRE FELLOW	Structural characterization of 3 essential genes from *M. tb*	Dr. Alamelu Raja
6.	Ms. Lakshmi R.	ICMR	Molecular studies on mycobacteria	Dr. Vanaja Kumar
7.	Mr. Sameer Hassan	ICMR-Biomedical Inf. Centre	Genome analysis of phages and viruses	Dr. Vanaja Kumar
8.	Ms. Yamuna N.	UGC	Classification and regression trees	Dr.P. Venkatesan
9.	Ms. Malini V.	ICMR	Functional characterization of FtsY, a signal recognition particle receptor from *M. tb*	Dr. Sujatha Narayanan
10.	Ms. Karthika K.D.	CSIR	Recombinant BCG based vaccine for TB	Dr. Sujatha Narayanan
11.	Ms. Suba S.	MSSRF	Characterization of Lipoproteins of *M.tb*	Dr.Sujatha Narayanan
12.	Mr. Srinivasan K.	NIH	Comparative genomics and pathogenesis of TB	Dr.Sujatha Narayanan
13.	Ms. Ahmed Kabir Refaya	ICMR	Mycobacterial transcriptional regulators in pathogenesis	Dr. Sujatha Narayanan
14.	M.V. Arunkumar	Lady Tata Memorial Trust	Gene regulation of mycobacteria	Dr. Sujatha Narayanan
15.	Ms.S. Priyadharshini	INSPIRE Fellow	Functional genomics of *M. tb*	Dr. Sujatha Narayanan
16.	Mr. Brijendra Singh	CSIR	Chemokine gene polymorphism and chemokine expression in PTB	Dr.P. Selvaraj
17.	Mr. Afsal K.	ICMR	Effect of vitamin D3 on innate and adaptive immunity in PTB	Dr.P. Selvaraj
18.	Ms. Nancy Hilda J.	UGC	Neutrophil mediated innate immune response in TB	Dr.D. Sulochana
19.	Mr. Jagadish Chandra Bose	ICMR-Biomedical Inf. Centre	Immunodominant epitopes against HIV subtype C	Dr. Luke Elizabeth Hanna
20.	Mr. Pawan Kumar N.	ICER	Pediatric TB	Dr. Luke Elizabeth Hanna
21.	Ms. Anuradha R.	ICER	Role of TLR in filarial pathology	Dr. Luke Elizabeth Hanna
22.	Mr. Narayanaiah Cheedarla	UGC	Comparative studies between HIV-1 and HIV-2 cases in Inda	Dr. Luke Elizabeth Hanna
23.	Mr. Jovvian George	ICER	Helminth Immunology	Dr. Luke Elizabeth Hanna

Staff registered (part-time) for their Ph.D. programme with the University of Madras, Chennai

Sl.No.	Name of the staff	Title of the Ph.D. thesis	Supervisor/Guide
1.	Ms. Amudha N.	Antimycobacterial compounds	Dr. Vanaja Kumar
2.	Ms. A.S. Shainaba	Phage based drug target identification and anti-mycobacterial drug discovery	Dr. Vanaja Kumar
3.	Mr. Anbalagan S.	Innate & adaptive immunity in HIV	Dr. Luke Elizabeth Hanna
4.	Mr. Harishankar M.	Role of vitamin D receptor promoter & 3'UTR gene variants on vitamin D modulated immune functions in TB	Dr.P. Selvaraj
5.	Mr. Rathinasabapati R.	Institutional repository for the Tuberculosis Research Centre	Dr.A. Amudhavalli, University of Madras
6.	Mr. Muthusamy M.	Antimicrobial and antimycobacterial agents	Dr. Vanaja Kumar
7.	Mr. Sekar L.	Survival analysis	Dr.P. Venkatesan
8.	Mr. Sivakumar S.	Molecular epidemiology of TB	Dr. Sujatha Narayanan
9.	Mr. Srinivasan R.	Spatial analysis	Dr.P. Venkatesan
10.	Mr. Sukumar B.*	Statistical methods for micro array data analysis	Dr.P. Venkatesan
11.	Ms. Vasantha M.	Structural equation modeling	Dr.P. Venkatesan

***** Ex-staff

N I R T - STAFF LIST

(As on 1 April, 2011)

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3. Dr.R.Balambal, M.D.,
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2. Ms. Niruparani Charles, M.A.,
3. Mr.K. Sankaran, M.Sc.,

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