AN IMMUNO-EPIDEMIOLOGICAL STUDY ON THE EFFECT OF NEONATAL BCG VACCINATION ON INTRACELLULAR KILLING OF *MYCOBACTERIUM TUBERCULOSIS BY* HUMAN MACROPHAGES

THESIS SUBMITTED TO
THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

BY

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For the Degree of Doctor of Philosophy

July 2009

DEPARTMENT OF EPIDEMIOLOGY
THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY
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This is to certify that the thesis entitled **'AN IMMUNO-EPIDEMIOLOGICAL STUDY ON THE EFFECT OF NEONATAL BCG VACCINATION ON INTRACELLULAR KILLING OF *MYCOBACTERIUM TUBERCULOSIS* BY HUMAN MACROPHAGES'** is a record of independent research work done by **Mr. C. MATHAN PERIASAMY** under my supervision and guidance during the period of his study from January-2004 to July-2009 for the degree of Doctor of philosophy at Department of Epidemiology, The Tamilnadu Dr. M.G.R Medical University, Chennai-32, South India. It has not previously formed the basis for any Degree, Diploma, Associateship or other similar titles.

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DECLARATION

I declare that the thesis entitled **'AN IMMUNO-EPIDEMIOLOGICAL STUDY ON THE EFFECT OF NEONATAL BCG VACCINATION ON INTRACELLULAR KILLING OF *MYCOBACTERIUM TUBERCULOSIS* BY HUMAN MACROPHAGES'** submitted by me for the degree of Doctor of Philosophy is a record of independent research work carried out by me during my study period from January-2004 to July-2009 under the guidance of **Dr. Manjula Datta**, at the Department of Epidemiology, The Tamilnadu Dr. M.G.R Medical University, Chennai-32, South India. It has not previously formed the basis for any Degree, Diploma, Associateship or other similar titles.

Signature of the Candidate

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ACKNOWLEDGEMENT

I express my sincere gratitude to all the persons who extended their helping hands from the beginning till the completion of this study. I acknowledge the contributions of the following persons who made it possible to complete the thesis.

My research supervisor and guide Dr. Manjula Datta, Professor and Head (Rtd), Dept of Epidemiology, The Tamilnadu Dr. M.G.R Medical University, Chennai for her guidance throughout the study. Her encouragement and personnal care have provided a good basis for this present thesis. Her comments have been of greatest help at all times and I thank her for correcting my mistakes with patience throughout my PhD program.

My co-guide Dr. M. Kannapiran, former Deputy Director, Tuberculosis Research Centre (ICMR), Chennai for giving me the opportunity to work with him and for providing me the best of facilities for all my cell culture experiments and encouragement throughout the work.

My thesis committee member Dr. V. D. Ramanathan, scientist-F, Department of Clinical Pathology, Tuberculosis Research Centre (ICMR), Chennai for his insightful advises, corrections and suggestions that have enriched my research work.

My mentor Dr. Major D. Raja, former Vice-Chancellor, The Tamilnadu Dr. M.G.R Medical University, for igniting a lasting interest in scientific research in my career.

Prof. Dr. K. Meer Mustafa Hussain, Vice-Chancellor and Dr. Sudha Seshayyan Registrar-in-charge, The Tamilnadu Dr. M.G.R Medical University for providing me the academic support to complete my study.

Dr. P.R. Narayanan, Former Director, Tuberculosis Research Centre, for permitting me to carry out my laboratory part of research work in Tuberculosis Research Centre.

Dr. Prema Gurumurthy, Former Head, Dept. of Biochemistry, Tuberculosis Research Centre and Dr. C.N. Paramasivan, Former Head, Dept of Bacteriology, Tuberculosis Research Centre for permitting me to utilize the laboratory facilities.

Dr. P. Venkatesan, Head, Department of Statistics and Dr. C. Ponnuraja, Scientist B, Department of Statistics, Tuberculosis Research Centre for carrying out the statistical analysis to my research work.

Mr. Ravi Datta, Executive Director, A Society for Primary Healthcare Intervention, Research and Education (ASPIRE), Chennai-87 & Programme Administrator, Kidney Help Trust, Chennai-18 for his encouragement, friendly advises and permitting me to utilize the transportation facilities in rural study area.

Dr. Jasmine S. Sundar, Dr. Vinodha Valladurai and Dr. Rajeshwari for their help in the clinical evaluation of the study subjects.

Dr. Jerard Maria Selvam, Head, Dept. of Epidemiology, The Tamilnadu Dr. M.G.R Medical University for his support and encouragement.

Dr. S. Joseph Maria Adaikalam, Lecturer, Dept of Epidemiology, The Tamilnadu Dr. M.G.R Medical University for his help and suggestions.

Dr. S. Kalpana and Mr. N. Valladurai, my seniors, for their scholarly interactions, suggestions and academic helps.

Dr. B.P. Shyamala, Dr. Malini, Dr. N.R. Somasekar, and Dr. N. Suganya, the epidemiologists who have helped me in many ways for this present work.

Mrs. Vaijayanthi and Mr. M. Suresh for their secretarial help, Dr. G. Srinivasan for his moral support and Mr. Vincent, Mr. Solomon and Mr. L. Srinivasan for their assistance throughout my study.

Mr. P. Ponnusamy, Mr. D. Rajkumar and Ms. R. Suganya for their help and cooperation.

Dr. S. Karthigayan for his help during the earlier days of my cell culture work.

Dr. Sarala Bai and Dr. Pandima Devi for their help and cooperation during the standardization experiments.

Dr. S. Manivannan and Dr. V. Narayan Rao, Researchers of Clinical pathology Dept, Tuberculosis Research Centre for their continuous moral support, constructive suggestions, help and cooperation during the entire study.

Dr. Anbarasu, Researcher in Immunology Dept, Tuberculosis Research Centre for his help and suggestions during ELISA experiments.

Dr. Prabha, Researcher in Immunology Dept, Tuberculosis Research Centre for her help and technical advises.

Mrs. Lalitha Victor, Mr. Jayaram, Mr. Raj Kumar, Ms. Chitra, Mr. Subramanian, Mr. Rajendiran, Mr. Manohar, Mr. Nambirajan, Mr. Chandran, Mr. Thiru Kumar, Mr. Azger and other staff members of Tuberculosis Research Centre for their help and co-operation.

Dr. S. S. Muthiah, Director/Senior consultant in human embryology for his constant external support.

Dr. Vel Murugan and Dr. Stalin for their support and academic helps.

Mr. A.N. Panneer Selvam, Mr. M.P. Selvakumar, Mr. Perumal, Mr. Palani and Mr. Pandiarajan, medical laboratory technicians who have helped me in tuberculin testing and blood collection procedures of the study.

Mr. Chandra Kesavan, Mr. Vijaya Kumar and Mr. Nedunchezian, social workers who have helped me in the organization of medical camps.

Mr. Punniakotti, Mrs. Amudha, Ms. Sumathy and all other staff members of Kidney Help Trust for their help in the recruitment and evaluation of rural study subjects.

All the library staff members of The Tamilnadu Dr. M.G.R Medical University, Tuberculosis Research Centre and Institute of Basic Medical Sciences for their kind cooperation during my literature collection.

M/s. Adyar Students Xerox Pvt. Ltd., Adyar, Chennai-20 for the physical shape of this thesis by their immense professionalism.

* * *

I gratefully acknowledge all my school teachers, UG/PG lecturers and other mentors who have helped me in my earlier studies.

* * *

I dedicate this work to my parents and I deeply acknowledge my beloved family members for their cooperation during the entire study.

C. MATHAN PERIASAMY

AFB	–	Acid Fast Bacilli
AIDS	–	Acquired Immuno Deficiency Syndrome
B cell	–	Bursa cell
BCG	–	Bacillus Calmette Guerin
CCL	–	Chemokine Ligand (C-C motif)
CD	–	Cluster of Differentiation
CDC	–	Centre for Disease Control
CFU	–	Colony Forming Unit
CXCL	–	Chemokine Ligand (C-X-C motif)
DOTS	–	Directly Observed Treatment Short Course
DPX	–	Distrene-80 Plasticizer Xylene
ELISA	–	Enzyme Linked Immuno Sorbent Assay
FACS	–	Fluorescence Activated Cell Sorter
HIV	–	Human Immunodeficiency Virus
HRP	–	Horse Radish Peroxidase
ICMR	–	Indian Council of Medical Research
ID	–	Intradermal
IFN γ	–	Interferon gamma
IL	–	Interleukin
IM	–	Intramuscular
IP	–	Intraperitoneal
IP10	–	Interferon-Inducible Protein-10
I-TAC	–	Interferon-inducible T-cell alpha chemoattractant
IUATLD	–	International Union Against Tuberculosis and Lung Disease
IV	–	Intravenous
LJ	–	Lowenstein Jensen
LMIT	–	Leukocyte Migration Inhibition Test

M. (genus)	–	*Mycobacterium*
MCP-1	–	Monocyte Chemotactic Protein-1
MDRTB	–	Multi Drug Resistant Tuberculosis
MHC	–	Major Histocompatibility Complex
Mig	–	Monokine induced by interferon-gamma
MIP1α	–	Macrophage Inflammatory Proteins 1 alpha
MIP1β	–	Macrophage Inflammatory Proteins 1 beta
MOI	–	Multiplicity of Infection
MPA	–	Meta Phosphoric acid
NK	–	Natural Killer
OADC	–	Oleic Acid Dextrose Complex
OT	–	Old Tuberculin
PBMC	–	Peripheral Blood Mononuclear Cells
PBS	–	Phosphate Buffered Saline
PCR	–	Polymerase Chain Reaction
PPD	–	Purified Protein Derivative
RANTES	–	Regulated upon Activation, Normal T-cell Expressed, and secreted
ROI	–	Reactive Oxygen Intermediates
RNI	–	Reactive Nitrogen Intermediates
RNTCP	–	Revised National Tuberculosis Control Programme
RPMI	–	Roswell Park Memorial Institute
SC	–	Subcutaneous
SNARE	–	Soluable N-ethylmaleimide-sensitive factor-attachment protein receptor
SOD	–	Superoxide dismutase
T cell	–	Thymus cell
TGF	–	Transforming Growth Factor
Th	–	T helper
TMB	–	Tetramethylbenzidine
TNF	–	Tumour Necrosis Factor

TST	–	Tuberculin Skin test
WHO	–	World Health Organization
XDR TB	–	Extremely (Extensively) Drug Resistant Tuberculosis

UNITS

µl	–	Micro liter
C	–	Celsius
g	–	Gravity
H^+ATP	–	Proton Adenosine Tri Phosphate
KDa	–	Kilo Dalton
mg	–	Milli gram
ml	–	Milli liter
mm	–	Milli meter
mM	–	Milli molar
nm	–	Nano meter
nMol	–	Nano moles
OD	–	Optical Density
Pg	–	Pico gram
TU	–	Tuberculin Units

STATISTICAL

CI	–	Confidence Interval
OR	–	Odds Ratio
RR	–	Relative Risk
SD	–	Standard Deviation

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- Introduction

Tuberculosis (TB) was the first disease to be declared a global emergency by the World Health Organization in 1993 (1), an honor not even enjoyed by small pox. It is an infectious disease caused by *Mycobacterium tuberculosis* mainly and sometimes other closely related *Mycobacterium* strains *M. africanum* and *M. bovis* which are commonly known as the TB-complex. The pathogen *M. tuberculosis* infects approximately 32% of the world human population and about 10% of the infected develop the disease (2).

Tuberculosis continues to be a major worldwide health problem and is the leading killer of youth and adults in developing countries, being responsible for more deaths than any other single infectious organism (1). Several prominent persons like John Keats, Leo Tolstoy, Alexander Graham Bell and Florence Nightingale have died of tuberculosis (3). Global prevalence of the disease increased greatly following the industrial revolution, with rapid urbanization and over crowding. HIV epidemic and the appearance of multidrug-resistant strains of *M. tuberculosis* (MDR-TB) have contributed significantly to the resurgence of TB (4).

Epidemiological projection by World Health Organization (WHO) shows that between the year 2002 and 2020 nearly 1 billion more people will be newly infected, 150 million get sick and 36 million die from tuberculosis if control measures are not strengthened (5).

In many developing countries, limited access to organized health care, inadequacies of tuberculosis control programs and more recently, the acquired immuno-deficiency syndrome (AIDS) pandemic have contributed significantly to the increase in tuberculosis case rates (6). Tuberculosis is preventable and in most cases can be cured, but various demographic and socioeconomic factors make both prevention and treatment difficult.

Bacillus Calmette–Guerin (BCG), an attenuated strain of *Mycobacterium bovis* is the only vaccine currently available against TB. This is the world's most widely used vaccine (~3 billion doses to date (7)) and being directed against the world's leading cause of infectious disease mortality, BCG is the most controversial vaccine in current use (8). The efficacy of BCG vaccine in the prevention of tuberculosis has shown considerable variation in different populations and trials (9). Results from those trials showed 0-80% of protection and particularly those from South India have shown no protection (9). However animal studies both in guinea pigs and mice have shown considerable protection (10), suggesting that the pathophysiological mechanisms after BCG in humans may be different from that in the animals.

Several attempts are being made to develop new TB vaccines. Though the BCG vaccine has been in use for nearly a century, the exact changes that it brings about in the humans and whether these changes are similar to or different from those brought about by natural sensitization have not been studied adequately. Clearly, understanding the mechanism by which the vaccine induces immunity would be important in being able to assess whether the new vaccines would be able to offer protection. This thesis is an attempt to address this issue with respect to the myco-bactericidal capacity of the macrophages and some cytokine responses.

- Literature Review

Tuberculosis has affected mankind for over 5000 years (11). The history of TB in earlier times has been traced through the analysis and interpretation of the evidence from human remains derived from archeological sites around the world. Evidence of the earliest affected human remains is from Italy dated around 5800 BC (12). The earliest pictorial evidence of TB probably refers to tuberculous lymphadenitis from Chinese manuscripts around 2700 BC (12). The ancient Asian literature Rig Veda makes reference to TB as early as 1500 BC as 'Rajayakshma' (King of diseases) (11).

Phthisis is a Greek term for tuberculosis; around 460 BC, Hippocrates identified phthisis as the most widespread disease of the times involving coughing up blood and fever, which was almost always fatal (11).

John Bunyan (1628-1688) was an English writer who referred to TB as the 'Captain of all these men of death' (12).

Rene Theophile Hyacinthe Laennec (1781 – 1826), a French physician first proposed the correlations between the pathological features of TB with its clinical symptoms (13).

Jean Antoine Villemin (1827 – 1892) was also a French physician who clearly established the infectious nature of tuberculosis. He demonstrated the transmission pattern in animal models but never identified the causative agent (13).

Robert Koch (1843 – 1910), a German physician and scientific investigator, concentrated his laboratory experiments in obtaining pure cultures of microorganisms. On 24th March-1882 he identified the causative agent of tuberculosis - '*Mycobacterium tuberculosis'* (13).

After identifying the agent, he set out to develop a cure for the disease and his approach was to stimulate the development of specific host resistance. In his animal experiments he observed that when a second injection of viable organisms was given to a guinea pig already infected with a subcutaneous focus of tubercle bacilli the animal showed healing at the site of second focus. In 1891, he proposed this mechanism of cell mediated immunity behind TB as 'Koch phenomenon'.

He introduced the heat-sterilized, concentrated filtrate of tubercle bacillus culture called 'Old tuberculin' as a therapeutic agent for tuberculosis, but it was proved to be an ineffective intervention (13). This early attempt to realize the role of tuberculin opened a new era in the field of immunology. Even though this type of reaction was described by Edward Jenner (1749-1823) nearly hundred years earlier (14), once Koch isolated the pure culture of the bacilli and demonstrated the immune response it was followed by many attempts to prepare a vaccine for TB.

M. tuberculosis is a straight or slightly curved rod shaped bacillus. It is an acid fast, aerobic, non-motile, non-capsulated and non-spore forming bacillus. It does not grow on ordinary media and requires enriched media with egg albumin. It grows slowly with generation time of about 16 hours. Colonies appear in 2-6 weeks on solid media. On Lowenstein Jensen's medium it forms dry, raised and irregular colonies typically described as the "rough, tough and buff" colonies. Optimum temperature for growth is 37^0C and optimum pH is 6.4 – 7. The bacillus can withstand weak disinfectants and can survive in a dry state for weeks.

The cell wall consists of a thick peptidoglycan and an arabinoglycan covalently linked by phospho-diester bonds. Its unusual cell wall, composed of high lipid content (up to 60%) called mycolic acid, a peculiar structure for this genus is likely responsible for this resistance and is a key virulence factor. Most virulent strains have a cord factor, a glycolipid of trehalose and mycolic acid that has several virulence properties. The sulfatides and sulfalipids in the membrane also play a role in the pathogenecity.

Ziehl - Neelson Staining technique is a standard technique used for smear microscopy. Acid fast bacilli are seen as pink bright rods while background is blue. Fluorescence staining methods are also available in which bacilli appear as bright rods against dark background. The important biochemical tests for its identification are Niacin test, Aryl sulfatase test, Nitrate reduction test and Catalase test.

M. tuberculosis causes natural infection in human beings by inhalation of small droplet nuclei of respiratory secretions containing the bacilli. It is a contagious disease that spreads directly or indirectly. The causative agent *M. tuberculosis* can survive for many years in soil or water and the entry is through aerosol / dust. When breathed in, the droplet nuclei containing 1–3 bacilli are able to reach the alveolar spaces. The larger droplets are carried up by the respiratory tract and are coughed up and destroyed.

The exact number of bacilli required to initiate infection is unknown though estimates vary from 5 – 200 inhaled bacilli (15). The inhaled bacilli become lodged in a distal respiratory bronchiole or alveolus. The tubercle bacillus does not appear to contain or produce a toxin. The various components of bacilli have been shown to possess different biological activities which may influence pathogenecity and immunity in disease (16).

Once deposited within the alveolar space, bacilli are ingested by resident pulmonary alveolar macrophages and are subsequently transported to hilar and mediastinal nodes. The bacilli are either destroyed or they multiply depending on innate killing profile of macrophages (17).

There are four potential outcomes of an infection by *M. tuberculosis* which are probably determined by the fate of the organism inside the macrophages (18).

The pathogen can

(i) infect a host and then be immediately eliminated – No infection. This is described as “exposure”.

(ii) become dormant indefinitely inside the host – Latent infection.

(iii) cause disease soon after infection – Primary tuberculosis.

(iv) cause disease after many years – Reactivation tuberculosis.

The pathogenic properties of *M. tuberculosis* can be ultimately linked to the intracellular fate of the organism. *M. tuberculosis* products that facilitate such intracellular outcomes may thus be defined as virulence factors.

Primary tuberculosis is the first manifestation caused in a human exposed to tubercle bacilli for the first time. At this site, the pathogen causes a lesion known as Primary focus or ‘Ghon’ focus. The primary infection is usually located at the lower lobe of the alveolus. Successive intracellular multiplication of bacilli is followed by the lysis of the infected macrophages leading to the enlargement of the primary lesion. During the stage of uncontrolled growth some bacilli are transported to lymph nodes where the pathological process is repeated. The initial lesion and its inflammed lymph nodes together form the ‘primary complex’ of tuberculosis.

During the first several weeks of infection, tubercle bacilli multiply and enter the blood stream through lymphatic channels and smaller pulmonary veins within the inflammatory exudates. Haematogenous seeding occurs most frequently at sites like brain, epiphyses of long bones, kidneys, vertebral bodies, lymph nodes and the apical-posterior areas of lungs. This primary infection progresses and tuberculosis occurs at the site as well as wherever it has disseminated and this is called Progressive primary tuberculosis; the

ultimate progression occurs in the host with no host response called disseminated miliary tuberculosis.

Unlike most other infectious diseases, in tuberculosis there is a delay between infection and disease that is extremely variable, ranging from a few weeks to a lifetime. Therefore, the development of active tuberculosis in someone known to have been previously infected raises the question whether this represents a reactivation of the initially infecting organism (endogenous reactivation) or a new strain of *M. tuberculosis* (exogenous reinfection). There have been some reports of exogenous reinfection, but these were thought to be restricted to HIV-positive patients who were highly immunocompromised (19). However, other findings suggest that reinfection may be more common than previously thought (20, 21, 22, 23).

Recent molecular biological techniques such as polymerase chain reaction (PCR) and restriction fragment length polymorphic (RFLP) analysis with IS6110 have illustrated the presence of simultaneous infection with multiple strains of *M. tuberculosis* (24, 25, 26, 27).

Macrophages are considered as the principal effector cells against *M. tuberculosis* but at the same time these cells act as the habitat for the multiplication of the bacilli and hence play a dual role in tuberculosis, promoting not only the protection against the disease but also survival of the pathogen (28). Bacilli which remain viable inside fused phagosomes use different mechanisms to escape from the post-phagocytic intracellular killing mechanisms of macrophage.

Phagocytosis

After evading the mechanical defenses of the upper respiratory tract, the droplet nuclei that contain viable *M. tuberculosis* bacilli make their way into the distal regions of the lung. Here they come into contact with the resident immune cell of the lung, the alveolar macrophage. Uptake of *M. tuberculosis* by macrophages represents the first major host–pathogen interaction in tuberculosis (29).

It has been demonstrated that complement receptors (30), mannose receptors (31), scavenger receptors (32) and others are capable of mediating this initial interaction. Most recently, attention has been focused on the role of toll-like receptors in mediating the uptake of mycobacteria by macrophages (33).

Phagosome – Lysosome fusion

In general most microorganisms that become ingested and engulfed by phagosomes are digested by lysosomal enzymes which are released in the phagosomes after fusion with the lysosomes (34). The lysosome is a highly complex organelle containing a multitude of enzymes within its own membrane that have the capacity to degrade a wide range of microorganisms. To persist in the host *M. tuberculosis* has evolved different mechanisms to inhibit the fusion of lysosome with phagosomes (35, 36). This intracellular survival rests upon its ability to arrest phagolysosome biogenesis, avoid direct cidal mechanisms in macrophages, and block efficient antigen processing and presentation (37, 38). *M. tuberculosis* interactions with the macrophage are dominated by the ability of the pathogen to prevent phago-lysosome biogenesis (39). *M. tuberculosis* produces ammonia in substantial amounts within the phagosomes. This weak base is presumed to be responsible for the inhibitory effect of phago-lysosome. In addition production of ammonia leads to alkalization of the compartment that may weaken the antimycobacterial activity of lysosomal enzymes which operate best at acidic pH (40). Another mechanism by which *M. tuberculosis* manipulates the phagosomes is to exclude H^+ (Proton) ATPases, thereby modulating acidic phagosomal compartment into one that is conducive to persistence (41, 42). Sulfatides of *M. tuberculosis*, or polyanionic trehalose 2-sulfates, have been proposed to be the products that inhibit phagolysosome formation (43). The ability of *M. tuberculosis* to inhibit the complement receptor – mediated Ca^{2+} signaling also contributes to inhibition of phagosome – lysosome fusion and promotion of survival (44). Studies on the molecular basis for the trafficking of intracellular vesicles

have identified the role of SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) and the small GTP-binding Rab family proteins that are involved in phago-lysosomal biogenesis (36, 45, 46).

Reactive Oxygen Intermediates

Phagocytosis by macrophages is accompanied by the oxygen-dependent process, 'Respiratory burst' in which a reduction of oxygen in a stepwise process involving the intermediate products superoxide anion, hydrogen peroxide and hydroxy radicals occurs. All these forms of activated oxygen have been implicated in antimicrobial activity (47). Sufficient effort has been focused on testing the role of the oxygen-dependent killing mechanism in defense against *M. tuberculosis*. Although there is evidence that these oxygen intermediates contribute to mycobacterial killing, their role in killing the phagocytosed *M. tuberculosis* remains unestablished (48, 49, 50, 51). *In vitro* experiments to reveal the role of peroxidative killing systems on antimycobacterial activity have shown that *M. tuberculosis* is susceptible to peroxidases (52).

Reactive Nitrogen Intermediates

Reactive nitrogen intermediates (RNI) generated from the metabolic pathway that begins with the oxidation of L-arginine in a variety of biologic functions (53). The protective role of reactive nitrogen intermediates is well established in murine system (54). Flynn JL *et al* and Chan J *et al* have reviewed the different mechanisms involved in the RNI mediated killing and the role of different genes such as noxR1, noxR3 and ahpC (35, 36). But the significance of RNI in the control of tubercle bacilli in human remains

controversial (55). This is because of the lack of an *in vitro* culture system that demonstrates high output of RNI from cultured human macrophages.

Antigen presentation

The recognition of infected macrophages by other immune cells such as CD4 and CD8 cells depends on presentation of mycobacterial antigens along with MHC receptors (35, 36). Presentation of mycobacterial antigens by macrophages involves distinctive mechanisms. First, MHC class II molecules present the recognized mycobacterial proteins to antigen specific CD4+ T cells. Second, MHC class I molecules are able to present mycobacterial proteins to antigen specific CD8+ T cells. This mechanism allows for the presentation of cytosolic antigens. Third, nonpolymorphic MHC class I molecules such as type I CD-1 molecules, which are expressed on macrophages are able to present mycobacterial lipoproteins to CD-1 restricted T cells. These mechanisms of antigen presentation by MHC enable the activation of a larger fraction of T cells at an earlier point of the infection (28).

CD4+ T lymphocytes

These are also called Th (helper) cells. Based on their cytokine secretion pattern, these CD4+ T cells are divided in to Th1 and Th2 cells. The importance of Th1 cells in tuberculosis host defense is their ability to secrete IFN- γ that plays a major role in TB immunology. Th2 cells secrete IL-4, IL-5 and IL-10 that are also playing a role in immunity to tuberculosis (29).

CD 8+ T lymphocytes

These cells are also known as Tc (Cytotoxic) cells. They recognize processed peptide fragments that are presented on cell surface in the context of MHC class I molecules. These cells can also secrete IFN- γ in a significant level. CD8+ T cells cause the lysis of infected target cells such as monocytes and macrophages and cause death of intracellular pathogens directly. These cells synthesize proteins called granulysin and perforin which can kill extra cellular *M. tuberculosis* directly (29).

Regulatory T cells

Regulatory T cells (T_{reg}), sometimes known as suppressor T cells, are a specialized subpopulation of T cells that act to suppress activation of the immune system. T_{reg} cells represent 5 to 10% of human circulating CD4 T cells. These regulatory T cells play a central role in the prevention of autoimmunity and in the control of immune responses by down-regulating the function of effector $CD4^+$ or $CD8^+$ T cells. The exact role of T_{reg} in *M. tuberculosis* infection and persistence is inadequately documented.

Recent studies have shown that these regulatory T cells are increased in blood/at the sites of disease and thereby suppress the immune response to *M. tuberculosis* (56, 57, 58). Much of recent research has been focused on the role of T_{reg} cells in the immunology of tuberculosis.

Cytokines are key mediator molecules in the expression of acquired immunity to tuberculosis (59, 60). When the tubercle bacilli are ingested by macrophages, they produce a characteristic pattern of cytokines. These cytokines have potent immuno-regulatory effects to mediate many of the clinical manifestations of tuberculosis. Cytokine cascade in tuberculosis includes pro-inflammatory cytokines and anti-inflammatory cytokines.

Pro-inflammatory cytokines

Tumour Necrosis Factor – α (TNF- α)

TNF- α is a potent mediator of inflammatory and immune functions (28). Human alveolar macrophages and mononuclear cells produce large quantities of TNF- α in response to mycobacteria or mycobacterial products (61). TNF- α plays a key role in granuloma formation, induces macrophage activation and has immunoregulatory properties (35). In tuberculosis patients, TNF- α production is present at the site of disease (62). Induction of TNF- α is also dependent on the macrophage CD14 molecule (63).

Interferon - γ (IFN- γ)

IFN- γ is a key cytokine in control of *M. tuberculosis* infection. It is produced by both CD4+ and CD8+ T cells, as well as by NK cells. The protective role of IFN- γ in tuberculosis is well established (64). It has many functions in the human defense system such as T and B cell modulation, antibody production regulation, cell surface antigen expression, macrophage activation and other important immune responses against the intracellular

multiplication of *M. tuberculosis* (28). Secretion of IFN-γ is potently stimulated by protein-peptidoglycan which is a major component of the *M. tuberculosis* cell wall membrane. It stimulates the production of TNF- α and both of these cytokines play a synergistic role in immune response chemotaxis (30).

Interleukin – 1 (IL-1)

IL-1 is mainly produced by monocytes, macrophages and dentritic cells. IL-1, along with TNF- α, plays an important role in the acute phase response such as fever and cachexia, prominent in TB. In tuberculosis patients, IL-1 is expressed in excess at the site of disease (65). IL-1 induces macrophages to produce pro-inflammatory cytokines and stimulates T-cell proliferation by regulating T-cell expression of IL-2 receptors and IL-2 production (66).

Interleukin – 2 (IL-2)

IL-2 is produced primarily by activated Th1 cells. It is a growth and differentiation factor for lymphocytes and NK cells. IL-2 has a pivotal role in generating an immune response by inducing an expansion of the pool of lymphocytes specific for an antigen. Therefore, IL-2 secretion by the protective CD4 Th1 cells is an important parameter and several studies have demonstrated that IL-2 can influence the course of mycobacterial infections, either alone or in combination with other cytokines (67).

Interleukin – 6 (IL-6)

IL-6 is produced by macrophages, T cells and monocytes. It has both pro- and anti-inflammatory properties. This cytokine has multiple roles in the

immune response, including inflammation, hematopoiesis and differentiation of T cells (68).

Interleukin – 12 (IL-12)

IL-12 is a key player in host defense against *M. tuberculosis*. It is mainly produced by phagocytic cells. It is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria. It has a crucial role in the induction of IFN- γ production (69).

Interleukin – 17 (IL-17)

IL-17 is a recently defined T cell cytokine that mediates tissue inflammation, and inflammatory response associated with tuberculosis (70). It is a potent inflammatory cytokine capable of inducing chemokine expression and recruitment of cells to parenchymal tissue. The IL-17 response is largely dependent upon IL-23 (71).

Interleukin – 18 (IL-18)

This pro-inflammatory cytokine shares many features with IL-1. This cytokine is also an IFN- γ inducing factor (72).

Interleukin – 23 (IL-23)

IL-23 is a regulatory cytokine that is important for the activation and clonal expression of Ag-specific CD4+ T cells in the draining lymph nodes of *M. tuberculosis*-infected lungs (73). IL-23 also promotes the differentiation of T cells into IL-17 secretion (74, 75).

Anti-inflammatory cytokines

Transforming Growth Factor - β (TGF - β)

This anti-inflammatory cytokine is produced in excess during tuberculosis and is expressed at the site of disease (76). This cytokine suppresses cell-mediated immunity by inhibiting the IFN-γ production in T cells. In macrophages it antagonizes antigen presentation, pro-inflammatory cytokine production and cellular activation (77).

Interleukin – 4 (IL-4)

IL-4 has the capacity to deactivate macrophage and to block T-cell proliferation by down-regulating the IL-2 receptor expression (78). So, excessive production of IL-4 contributes to suppression of the immune response in tuberculosis patients.

Interleukin – 10 (IL-10)

IL-10 is produced by macrophages and T cells. This cytokine decreases the pro-inflammatory cytokine response by down-regulating the production of IFN-γ, TNF-α and IL-12 (79). IL-10 also suppresses antigen-specific T-cell proliferation by down-regulation of macrophage class II MHC expression (80).

Chemokines

Chemokines are small molecular mass chemotactic cytokines (8 -14 KDa) that mediate constitutive and inflammatory recruitment of leukocytes from the blood in to tissues (81). Approximately 40 chemokines have been identified and are grouped into four structurally related families determined

by the number of amino acids (82). A number of chemokines have been investigated in tuberculosis. CXCL8 (IL8) is a well studied chemokine which attracts neutrophils, T-lymphocytes and monocytes. CCL2 (MCP-1) and CCL5 (RANTES) also play a major role in tuberculosis. Other chemokines include CXCL9 (Mig), CXCL10 (IP10), CXCL11 (I-TAC), CCL3 (MIP1α) and CCL4 (MIP1β) that are essential for recruitment of cells.

Apoptosis

Apoptosis is the process of programmed cell death that has been recognized as a component of protective host response to intracellular infections (83). Apoptosis of alveolar macrophages has now been demonstrated to be a common response to intracellular infection by *M. tuberculosis* in murine (84) and human experiments (85, 86, 87). The role of apoptosis in response to *M. tuberculosis* infection has only recently been appreciated and it's implication for *in vitro* experimentation and for the pathology of tuberculosis remains to be explored (88).

Tuberculosis is a major cause of illness and death worldwide, especially in Asia and Africa. An estimated one third of humanity (approximately two billion people) is infected with *M. tuberculosis*. Amongst those carrying the pathogen, around 8 million persons come down with clinical disease every year and out of these, about 1.6 million die. Globally, 9.27 million new cases occurred in 2007. India, China, Indonesia, South Africa and Nigeria rank first to fifth respectively in terms of absolute numbers of cases (2). The estimated number of new tuberculosis cases by country in the year 2007 is shown in fig-1.

Tuberculosis is a major public health problem in India. Each year nearly 2 million people in India develop TB, of which around 0.87 million are infectious cases. It is estimated that annually around 3,30,000 Indians die due to TB (89). The estimates of epidemiological burden in India are given in table-1.

Table – 1. Epidemiological burden of tuberculosis in India (2)

Global rank by no. of cases	1
Incidence (all new cases/100 000 pop/yr)	168
Prevalence (all cases/100 000 pop)	283
Mortality (deaths/100 000 pop/yr)	28
HIV + incident TB cases (% of all TB cases)	5.3
Of new TB cases, % MDR-TB	2.8
Of previously treated TB cases, % MDR-TB	17

India accounts for one-fifth of the global incidence of TB and 2/3rd of South-East Asia (Fig-2) (90). This makes India the highest TB burdened country in the world (89).

Resistance to anti-TB drugs can occur when these drugs are misused or mismanaged. This resistance leads to more severe forms of TB such as MDR and XDR TB (91). Multidrug-resistant TB (MDR TB) is TB that is resistant to at least two of the best anti-TB drugs, Isoniazid and Rifampicin. These drugs are considered first-line drugs and are used to treat all persons with TB disease (91). Extensively drug resistant TB (XDR TB) is defined as the TB which is resistant to Isoniazid and Rifampin, plus resistant to any fluoroquinolone and at least one of the three injectable second-line drugs (i.e., Amikacin, Kanamycin, or Capreomycin) (91).

The increase in tuberculosis incidence is strongly associated with the prevalence of HIV infection (92). It is seen thus, that there is a lot more to be done in order to control TB worldwide.

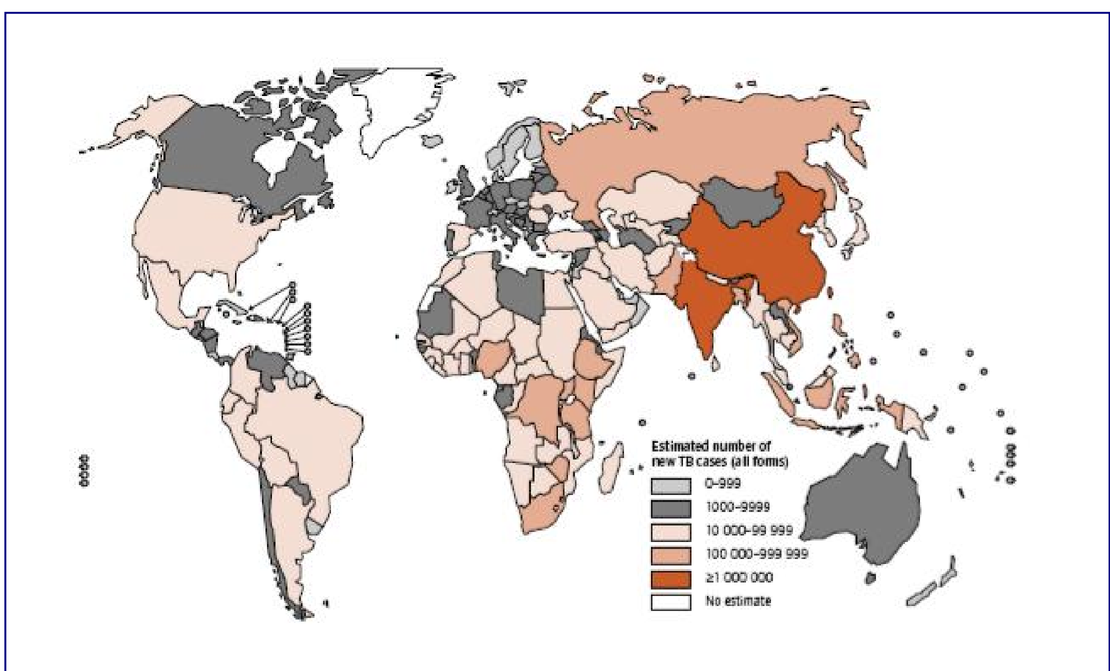


Fig-1. Estimated number of new TB cases by country in 2007 (2)

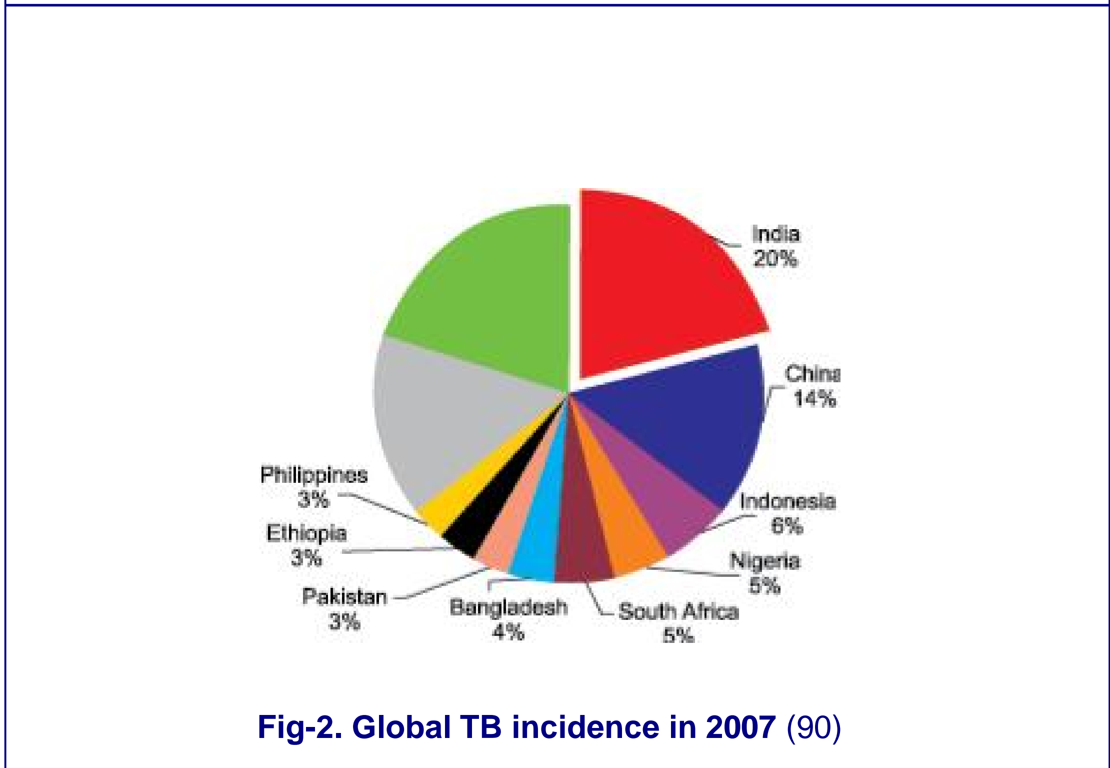


Fig-2. Global TB incidence in 2007 (90)

In 1896, Albert Calmette a French physician was appointed as the director of the Pasteur Institute of Lille, and the main public problem which he had to contend with was tuberculosis. Camille Guerin, a veterinary surgeon was appointed to assist Calmette in 1897 and drawn to research on anti-tuberculosis vaccine (93).

In 1903, Von Pirquet recognized the potential value of tuberculin for the detection of persons who were previously infected with *M tuberculosis,* using skin test by hypersensitivity reaction (93). Calmette associated tuberculin sensitivity with immunity and planned to prepare a live vaccine after considering that hypersensitivity resulted from the infection with live tubercle bacilli. They observed that addition of ox bile to the medium leads to the lowering of the virulence of the organism and this observation led them to take their long-term attempt to prepare a vaccine from live attenuated bacilli.

They started their subculture work with a virulent bovine strain of tubercle bacillus in 1908. They cultured the bacilli on bile, glycerin and potato medium and then preceded to subculture at three weeks intervals. By 1919 after about 230 subcultures carried out for 11 years they succeeded to get *M. bovis* bacillus which failed to produce progressive disease in animals (93).

In 1921, first human administration of BCG was carried out by Weil Halle to an infant through oral route (7). Between 1924 and 1928 more than one lakh infants were vaccinated without any complications. The method of BCG vaccination was therefore proved to be safe (93).

After the reports of successful BCG vaccination in 1921, cultures of BCG were delivered for propagation in laboratories all over the world. The original *M. bovis* BCG vaccine strain was developed into several different sub strains which have been used for production of BCG vaccine. Between 1924 and 1926, 34 countries received BCG cultures from Pasteur institute (Table – 2) and later, many other countries were also reported to have received BCG cultures from Paris (94).

Table – 2. List of Countries to which BCG cultures were distributed from Pasteur Institute (1924-1926)

Names of Countries				
Algeria	Canada	Italy	Peru	Switzerland
Argentina	Columbia	Japan	Poland	Syria
Austria	Czechoslovakia	Lithuania	Romania	Uruguay
Belgium	Germany	Mauritius	Russia	USA
Bolivia	Greece	Mexico	Scotland	Venezuela
Brazil	Holland	Norway	Spain	Yugoslavia
Bulgaria	Hungary	Palestine	Sweden	

Many of these BCG substrains are no longer in use. Among the BCG substrains produced and used the following BCG substrains; BCG Moreau, BCG Tokyo, BCG Copenhagen, BCG Danish, BCG Tice, BCG Montreal, BCG Connaught, BCG Glaxo and BCG Pasteur are of historical interest.

BCG Moreau

In 1924, Dr. Moreau received this strain from Pasteur Institute and was propagated until 1950s and was freeze-dried. Later in 1960 a batch was brought to Copenhagen. A small batch of vaccine was produced on a request of WHO and nominated as a primary seed lot (94).

BCG Tokyo

In 1925, Dr. Shiga transported a seed culture from Pasteur Institute to Tokyo and subjected to large-scale production in 1946. It has been used as a freeze-dried vaccine since 1950 (94).

BCG Copenhagen (Gothenburg)

Dr. Wassen, a colleague of Calmette, brought a subculture to Sweden in 1926. Later Statens Serum Institute took over production of this substrain and named it as BCG Copenhagen (94).

BCG Danish

In 1931, Statens Serum Institute received a BCG strain. In 1960 the batch-1331 was freeze-dried, and after extensive clinical testing this batch was adopted as BCG Danish-1331 (94).

BCG Tice

In 1933, Dr. Tice sent Dr. Rosenthal to the Pasteur Institute with the aim of starting vaccine production in USA. The substrain he received from Paris is known as BCG Tice (94).

BCG Montreal

In 1937, Dr. Frappier received a BCG strain from Pasteur Institute which was developed into BCG Montreal (94).

BCG Connaught

In 1948, Dr. Brown received a bile-potato culture and the freeze-dried batch 140 was named as BCG Connaught (94).

BCG Glaxo

In 1954, Glaxo was supplied a culture from the 1077th transfer of BCG Danish from Statens Serum Institute. A freeze-dried master seed-lot from this is now called BCG Glaxo (94).

BCG Pasteur

In 1961, the present Pasteur strain was derived from a single colony (out of thirty colonies) that yielded cultures. This strain 1173-P was freeze-dried and the second batch is termed as BCG Pasteur or 'French' strain (94).

Present manufacturers

The major producers of BCG vaccines in international market are as follows (95);

Pasteur – Merieux – Connaught
Danish – Statens Serum Institute
Evans Mediva (which has taken over old Glaxo vaccine)
Japan BCG laboratory – Tokyo
The BCG Laboratory, Chennai – India

Approximately 100 million children receive BCG annually throughout the world today (95). Most of the countries follow the Universal Immunisation Programme which recommends only a single dose of BCG given at birth or at earliest contact with a health service.

Tuberculins are extracted proteins from the *Mycobacterium* species. The first tuberculin test material was developed by Robert Koch and called as Old Tuberculin (OT) which was a crude extract (96). But the potential value of the tuberculins for the detection of infected individuals was identified by Von Pirquet in 1907 (96). The intradermal injection of the tuberculins for the diagnosis of infection was introduced by Charles Mantoux in 1908 (96). The PPD (Purified protein derivative) materials were standardized by Florence Seibert in 1934 at Phipps Institute in Philadelphia (96).

The Tuberculin skin test is performed by injecting 0.1 ml of tuberculin purified protein derivative (PPD) into the inner surface of the forearm (91). The injection is administered with a tuberculin syringe, with the needle bevel facing upward.

Tuberculin sensitivity is a delayed type of hypersensitivity reaction in the form of induration at the test site in sensitized host (97).

The skin test reaction is usually read between 48 and 72 hours after administration. The reaction is measured in millimeters of the induration (palpable, raised, hardened area or swelling) only and not the erythema (redness). The diameter of the indurated area is measured across the forearm (98).

As on today, the only accepted material for the use of tuberculin skin testing is PPD (96). Different doses of PPDs are available in market. The inner or

volar aspect of the forearm site is generally used for the administration of tuberculin skin test.

Some of the widely used specific tuberculins are mentioned below (96);

PPD-S, PPD-T, RT-23	*M. tuberculosis*
PPD-B	*M. intracellulare*
PPD-G, RS-95	*M. scrofulaceum*
RS-10	*M. avium*

Atypical mycobacteria are obligate aerobes that can be found in the environment, soil, water, vegetables, and even in domestic animals and dairy products. They are also referred as Non-tuberculous mycobacteria (NTM). They resemble *M. tuberculosis* but exhibit number of atypical characters. They grow much faster than *M. tuberculosis.*

A study conducted in South Indian BCG trial area to identify the prevalence of non-tuberculous mycobacteria has demonstrated 8.6% in the rural trial area and 7.6% in the adjacent areas. In Chennai the prevalence was 4.5%. Species level identification has revealed both pathogenic and non-pathogenic forms of non-tuberculous mycobacteria (99).

Different environmental mycobacterial species induce different immunological responses and these responses may beneficially increase the immune response or may decrease the immune response against the infection with *M. tuberculosis* (100).

There are many different species of atypical mycobacteia and those that cause atypical mycobacterial infections include *M. avium-intracellulare, M. scrofulaceum, M. asiaticum, M. kansasii, M. marinum, M. ulcerans* etc. Non-pathogenic atypical mycobacteria include *M. terrae* complex, *M. flavescens, M. gordonae* etc.

Clinical trials and case-control studies

Summary of published results

In the mid-1930s the safety and efficacy of BCG vaccines were epidemiologically assessed by properly conducted trials. Vaccine efficacy is expressed as the per cent reduction in risk of disease in vaccinated individuals when compared to comparable non-vaccinated individuals (101). A list of controlled trials that were begun in 1930s and the well-known trials are shown in table – 3.

BCG vaccination came into widespread use in the 1950's and 1960's with the support and encouragement from World Health Organization.

Despite the nature of results of randomized controlled trials including Chingleput trial, BCG vaccination continues to be recommended by WHO as a part of immunization programme for infants in the majority of the countries.

So, the epidemiologists and researchers decided to assess the efficacy of the vaccine retrospectively by estimating the effect of TB rates on who had BCG at birth or at campaigns.

A list of ten well-known case control studies is shown in table – 4.

Table – 3. Clinical trials of BCG vaccine (7)

S. No	Trial	Country	Year	Description	RR
1	Saskatchewan Trial (102)	Canada	1933	609 Saskatchewan-American infants were included Incidence among Vaccinated - 6/306 Incidence among Control - 29/303	0.20
2	Aronson trial (103)	USA	1935	262 American individuals were included Incidence among Vaccinated - 4/123 Incidence among Control - 11/139	0.41
3	Rosenthal-chicago trial-1 (104)	USA	1937	3381 Chicago infants were included Tice BCG was used Incidence among Vaccinated - 17/1716 Incidence among Control - 65/1665	0.25
4	Rosenthal-chicago trial-2 (105)	USA	1941	451 Chicago newborns were included Incidence among Vaccinated - 3/231 Incidence among Control - 11/220	0.26

5	Muscogee trial (106)	USA	1947	4839 Muscogee school children were included Tice BCG was used First trial supported by WHO+USPHS Incidence among Vaccinated - 5/2498 Incidence among Control - 3/2341	1.56
6	Puerto Rico trial (107)	USA	1949 to 1969	77972 children and adults were included Study was supported by USPHS Birkhaug BCG was used Incidence among Vaccinated - 186/50634 Incidence among Control - 141/27338	0.71
7	Muscogee and Russel trial (108)	USA	1950 to 1970	34567 individuals with >5 years were included Study was supported by USPHS BCG obtained from Rosenthal was used Tuberculin test and X-ray examination were carried out before vaccination Incidence among Vaccinated - 27/16913 Incidence among Control - 29/17854	0.98

8	MRC trial (109)	UK	1950 to 1967	26465 British school children and school leavers with 14-15 years were included Tuberculin test and X-ray examination were carried out before vaccination BCG Copenhagen was used Incidence among Vaccinated - 62/13598 Incidence among Control - 248/12867	0.24
9	South India-Madanapalle trial (110)	India	1950	About 21,000 villagers were included and TST performed with 5 TU of Danish PPD The non-reactors were included and divided in to vaccinated and unvaccinated individuals. Out of 10877 non-reactive subjects Incidence among Vaccinated - 33/5069 Incidence among Control - 47/5808	0.80
10	Chingleput major trial (111)	India	1968	505 TB cases among vaccinated 499 TB cases among unvaccinated	1.01
Overall RR (95% Confidence Interval)				0.49 (0.34 – 0.70)	

Table – 4. Case Control studies of BCG vaccine (7)

Study Reference	TB cases		Controls		Odds ratio
	BCG	No BCG	BCG	No BCG	
Putrali 1983 (112)	59	44	281	131	0.63
Shapiro 1985 (113)	38	140	247	73	0.84
Young & Hershfield 1986 (114)	35	36	163	50	0.39
Myint 1987 (115)	162	149	977	559	0.62
Miceli 1988 (116)	50	125	519	356	0.27
Packe & Innes 1988 (117)	62	46	336	96	0.36
Houston 1990 (118)	65	78	148	103	0.58
Sirinavin 1991 (119)	57	18	189	18	0.17
Rodrigues 1991 (120)	57	54	356	199	0.51
Patel 1991 (121)	57	82	140	156	0.79
Overall OR (95% Confidence Interval)			0.50 (0.39 – 0.64)		

Several different hypotheses were proposed for the failure of BCG vaccine to protect against TB in the field trials (8, 101, 122) and are reviewed below.

Previous exposure with atypical mycobacteria

This hypothesis is oldest and still one of the most popular explanation for the failure of protective efficacy offered by the BCG vaccination. The previous sensitization and infection with environmental atypical mycobacteria provide some protection against *M. tuberculosis* and then the effect of later BCG is partially masked. Numerous species of mycobacteria are found in soil and water and many of human beings are sensitized to these bacteria and this hypothesis would appear to be plausible. The fact that atypical mycobacteria (NTM) were isolated from 8.6% of sputum specimens collected from the study area of Chingleput BCG trial lends support to this theory (99).

Animal studies have provided additional evidence that sensitization with environmental mycobacteria may have a direct antagonistic effect on BCG vaccination. Mice pre-sensitized with *M. avium* or with cocktails of *M. avium*, *M. vaccae* and *M. scrofulaceum* developed antimycobacterial responses that control the multiplication of BCG, thereby reducing its protective efficacy against TB (123). Sensitization with *M. avium* or *M. fortuitum* before vaccination with BCG also showed a modulatory effect on the protective efficacy of BCG against experimental TB in guinea pigs (124). These results strongly suggest that prior exposure to live environmental mycobacteria primes the host immune system against mycobacterial antigens shared with BCG and that recall of this immune response on vaccination results in accelerated clearance of BCG and hence decreased protection against TB.

Differences between BCGs

The differences in the potency, immunogenecity and dose of individual vaccine strains are also considered as the most important hypotheses for the failure of BCG vaccine. Similar vaccines showed very different efficacies in the Chingleput and British trials against tuberculosis. In Chingleput BCG trial two different vaccines of BCG the French and Danish, with a high dose (0.1mg/0.1ml) and a low dose (0.01 mg/0.1 ml) were used and showed similar protection against tuberculosis (109, 111).

Differences in natural history of infection and disease

According to this hypothesis, the variation in efficacy could be related to differences in risk of infection, differences in *M. tuberculosis* or to differences in pathogenesis of disease. Studies in guinea pigs have shown that the protection imparted by BCG vaccines may differ according to the exposure of strain of *M. tuberculosis* (125).

Variations in host genetics or nutrition

This hypothesis depends upon gender difference and other genetic differences that are believed to play a role in the differential protection offered by BCG (122). There is no direct evidence that the variation in the protection imparted by BCG is related to genetic factors in human populations. However large scale studies at molecular level may provide vital information.

Differences in nutritional status

As nutritional status affects the functioning of the cellular immune system, it might be expected that poor nutritional status would adversely affect the protective efficacy of BCG vaccination (122). To support this hypothesis, large scale epidemiological studies are needed.

Methodological differences in the trials

The mathematical and statistical differences between sample size, field methods and other sampling differences were also considered for the variable results in the protection offered by the BCG (122).

- Scope and Plan of work

BCG has been in use for over six decades in the prevention of tuberculosis in several countries. It is not only an extensively used but also one of the most extensively studied vaccines because of the controversy over its efficacy.

Earlier animal studies carried out to reveal the immune response elicited by BCG have provided only limited information about its mode of action (126). But the understanding of the pathogenesis of tuberculosis has been enhanced through comparisons of the course of infection in vaccinated and non-vaccinated animals. Several animal models were used for the assay of protective effect of BCG (10). Some of the earlier animal model experiments have showed considerable protection of BCG among mice (127,128,129, 130), guinea pigs (131,132,133), Rhesus monkeys (134,135,136) and Bank voles (137, 138). These studies have significantly increased our understanding of the aetiology, virulence and pathogenesis of the disease (139, 140).

After the reports of 7½ years follow-up of BCG trial in rural South India in early 80’s, several hypotheses were proposed to explain those unexpected findings (8) and these hypotheses were tested through animal experiments to explain the failure of BCG (141, 142, 143). Rook, Stanford and associates have also correlated the immune response of BCG along with the immune response elicited by exposure to environmental mycobacteria. They have suggested that the exposure to environmental mycobacteria results in two types of cell-mediated immunity; Listeria type and Koch type, both of which produce reactive tuberculin responses. In the former, the immune response was enhanced by the subsequent BCG vaccination whereas in the latter, the immune response opposed the protective effect of BCG (100).

Earlier studies that have attempted to reveal the immune response of BCG vaccination were focused on leukocyte migration analysis (144, 145). Later, a study carried out to reveal the influence on monocyte/macrophage functions has demonstrated an increased anti-mycobacterial activity of the mononuclear phagocytic cells after BCG vaccination (146). This study was further extended by the Tuberculosis Research Centre, Chennai, on Indian subjects to compare with British subjects and had showed that no significant differences in the patterns of macrophage induced killing of *M. tuberculosis* among Indians either before or after BCG vaccination (147). But in these studies the influence of tuberculin response was not studied.

In another study carried out by Tuberculosis Research Centre, Chennai, the cell-mediated immune response induced by BCG was revealed by tuberculin skin test, lymphocyte proliferation and cytokines secretion level among South Indian subjects of Chingleput BCG trial area. The study has showed that BCG does cause skin test positivity but the lymphocyte proliferation remained unaltered by BCG vaccination. The production of IFN-γ was significantly higher among the tuberculin reactors when compared to the non-reactors and the BCG vaccination did not change the level of IFN-γ (148).

Later, the publication of 15-year follow-up of South Indian Chingleput BCG trial also showed no difference in the incidence of tuberculosis in those given vaccine or placebo (149). The reason for the failure of BCG to protect against tuberculosis in these populations has been the subject of much research.

Importantly, BCG vaccines consistently give good protection against childhood manifestations of TB when administered before sensitization with

environmental mycobacteria (150). However, as the activity of the neonatal vaccination wanes after 10-12 years (151), the incidence of pulmonary TB increases in adolescence (152). The mechanism underlying the gradual loss of effectiveness of BCG (neonatally vaccinated) as the individual reaches 10 to 15 years of age is poorly understood. Moreover these adolescent/young adult population are highly sensitized due to the combination of BCG vaccine, environmental mycobacteria and in some cases with latent TB infection. Unvaccinated subjects also develop natural immunity with time, through the exposure with environmental mycobacteria.

The exact mode of action elicited by neonatal BCG vaccination and the influence of natural sensitization (reflected by tuberculin skin test) in adolescent/young adulthood have not well understood and not studied adequately.

So, an immuno-epidemiolocal study on the mode of action of existing BCG vaccine and influence of natural sensitization, particularly on macrophage cells in different population will enhance the understanding of BCG vaccine and provide an essential background for the development of new TB vaccines. This tentative first step is taken in this thesis.

Present study was designed in a way to make an attempt to obtain the answers for the following questions;

- Can we evaluate the immune response elicited by neonatal BCG vaccination by comparing the tuberculin non-reactors of BCG vaccinated and unvaccinated by the capacity of macrophages killing profile?

- Not all people vaccinated with neonatal-BCG show a positive response to tuberculin during adolescence or early adulthood. Can this dichotomy-tuberculin response (reactive and non-reactive) be an indication of the killing capacity of the macrophages?

- If the macrophage response in the tuberculin reactors without BCG vaccination is similar to that in the tuberculin reactors with BCG vaccination, would this indicate that a tuberculin-reactivity is only an indication of new infection and not a marker of BCG?

- Are the responses of macrophages from BCG vaccinated tuberculin non-reactors significantly different from non-vaccinated tuberculin reactors?

These information on the existing BCG vaccine may provide a vital background for the development of new TB vaccines. Several attempts were focused on recombinant vaccines and on booster TB vaccines (153). These information about the existing vaccine particularly in endemic countries like India may provide a better understanding for the rational designing of new or recombinant vaccines.

- Aims and Objectives of the study

- To assess the proportion of tuberculin response among the study population with and without prior BCG vaccination in three different geographical locations

- To analyze the influence of various risk factors associated with tuberculin skin test reactivity in the study population

- To evaluate the immune response elicited by neonatal BCG vaccination in healthy young adults by comparing BCG vaccinated and unvaccinated tuberculin non-reactors as measured by the capacity of the macrophages for killing *Mycobacterium tuberculosis* H37Rv

- To evaluate the effect of tuberculin responses (reactive and non-reactive) after BCG vaccination, in the macrophage functions by comparing the vaccinated tuberculin reactors with vaccinated tuberculin non-reactors

- To compare the macrophage responses in the tuberculin reactors without BCG vaccination with the tuberculin reactors with BCG vaccination

- To compare the effect of neonatal BCG vaccination with the effect of natural sensitization with mycobacteria by comparing BCG vaccinated non-reactors with unvaccinated reactors through anti-mycobacterial activity of macrophages

This is essentially formative research to see how the macrophages behave in the presence of BCG vaccination and natural exposure-sensitization of the host with mycobacteria. Also to see if those in whom the reaction size wanes after vaccination are different from those in whom the reaction size does not wane.

Hypothesis of the study is “Neonatal BCG vaccination does not influence the antimycobacterial activities of human macrophages in their adolescent/young adulthood. The anti-mycobacterial capacity of their macrophages depends on natural sensitization of the host with mycobacteria as reflected through tuberculin skin test.”

- Materials, Methods and Results

Present study was planned and designed as a cross–sectional study to perform an Immuno-epidemiological investigation on the effect of neonatal BCG vaccination and the effect of natural exposure and on the macrophage killing profile.

The study includes three chapters;

1. Epidemiological field work which included selection of study area, screening of study population, determining related risk factors and performing tuberculin skin test (Fig – 3).

2. Standardization of laboratory experiments including PBMC viability, and Quantification of *M. tuberculosis* H37Rv.

3. Immunological laboratory experiments including Measurement of phagocytosis, Superoxide Dismutase assay, Glutathione assay, whole blood assay (Cytokines estimation) and intracellular growth kinetics of *M. tuberculosis* H37Rv (Fig – 4).

Sampling

This cross-sectional study includes complex and lengthy procedures both in epidemiological and immunological parts. Considering the convenience, feasibility and resources available the convenient sampling method was applied (154).

Ethical Committee approval

The design and methodology of the entire study was approved by the institutional ethical committee of The Tamilnadu Dr. M.G.R. Medical University (Annex-I).

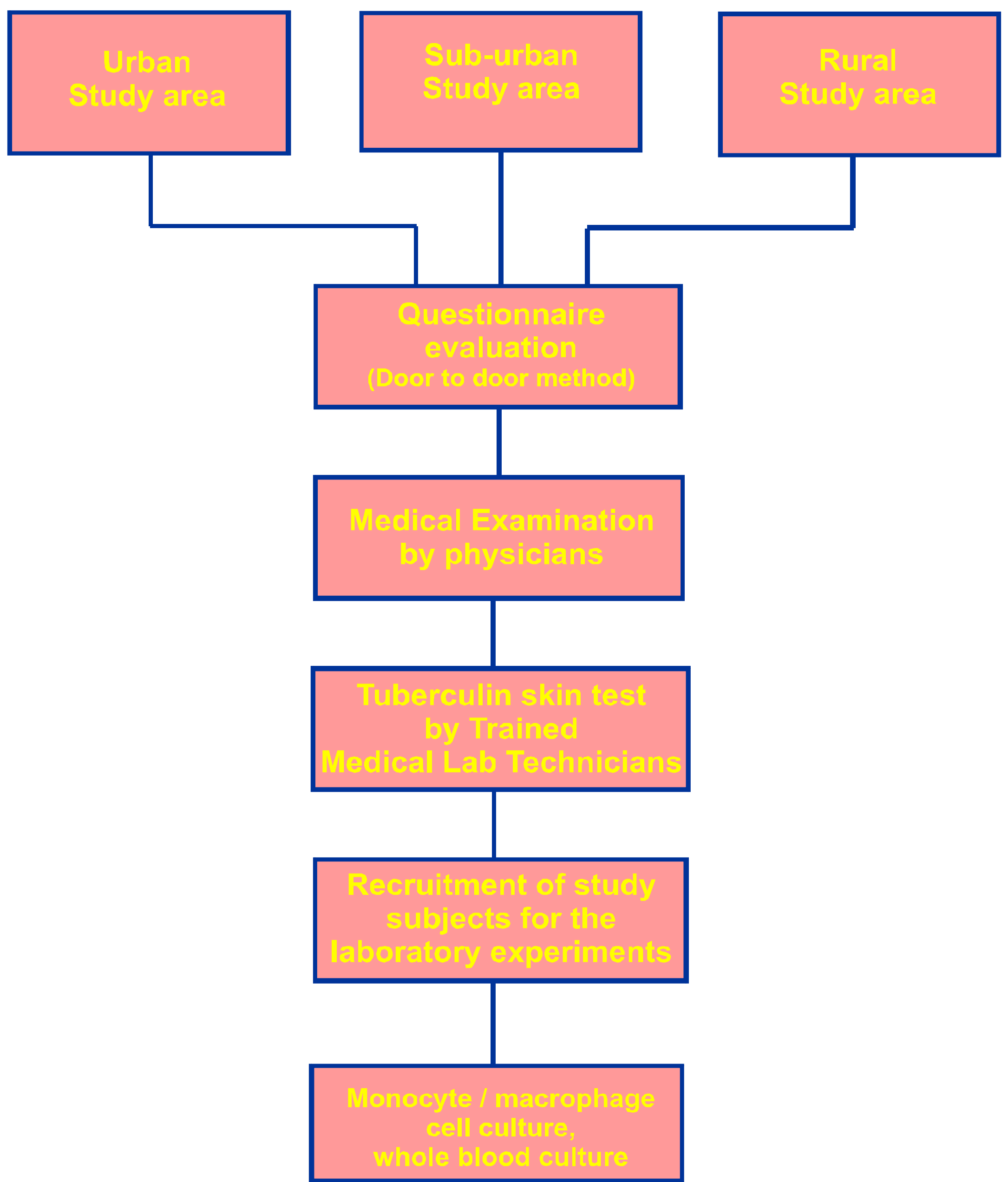


FIG.3. PROTOCOL FOR EPIDEMIOLOGICAL FIELD WORK
(Selection of study subjects)

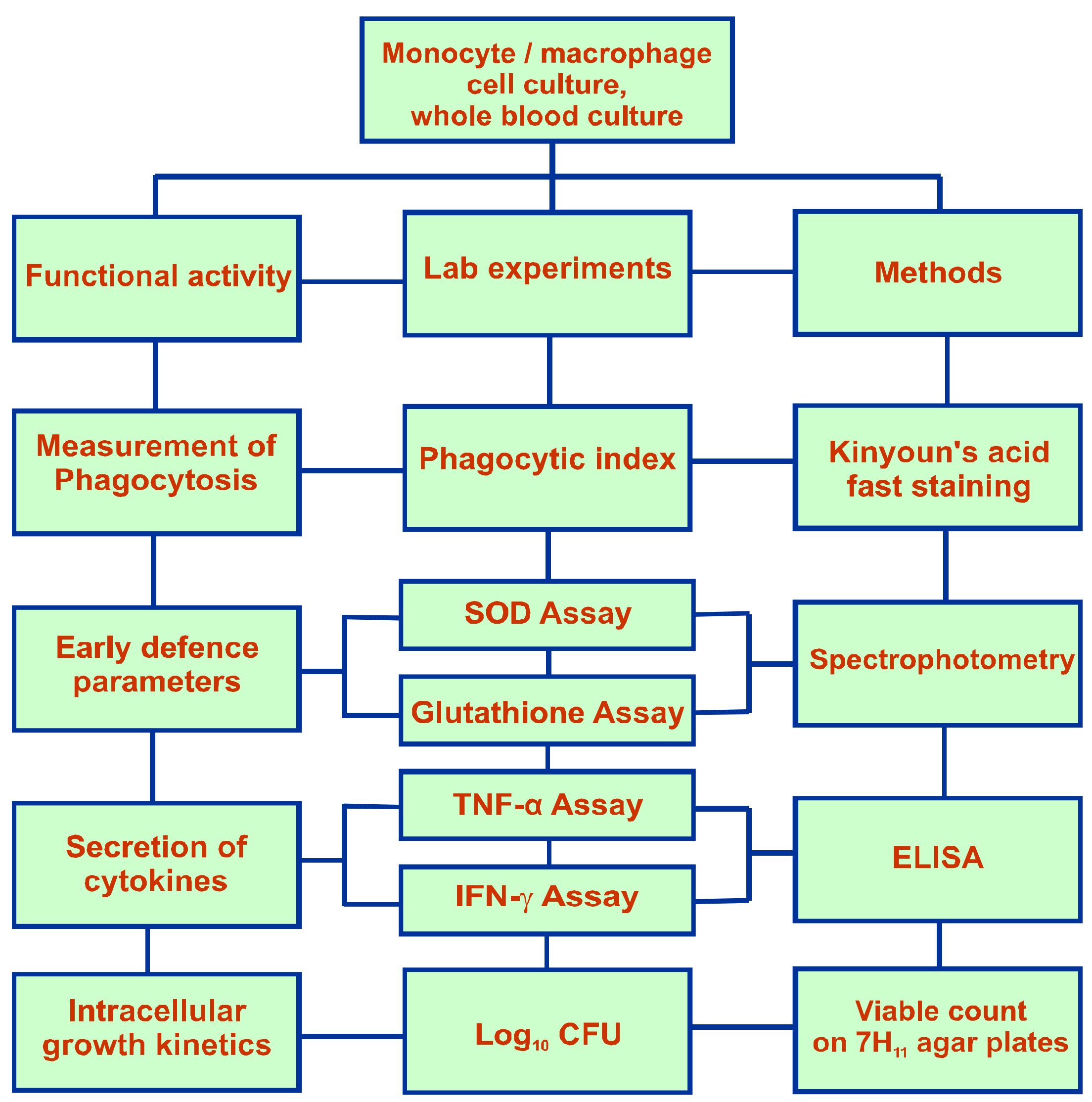


FIG.4. PROTOCOL FOR IMMUNOLOGICAL LABORATORY WORK
(Elucidation of macrophage responses)

- Chapter – I : Epidemiological field work

1. Urban Chennai

Chennai, formerly known as Madras, is the capital of the South Indian state of Tamil Nadu. With an estimated population of 7.06 million, it is the fourth largest metropolitan city in India. Chennai is the third largest commercial and industrial centre in India. The city faces problems with water shortage, traffic congestion and air pollution. Chennai has a diversified economic base anchored by the automobile industry, software services, hardware manufacturing, healthcare and financial services industries. The average literacy rate is 80.14% (155).

2. Sub-urban Pallavaram

The second study area Pallavaram is a sub-urb of Chennai city and a municipality in Kancheepuram district of Tamilnadu. It is a residential cum industrial area with numerous leather tanneries and chemical industries. The study area is surrounded by numerous stone crushers. The total population is about 1,50,000 and most of them are laborers in those companies/industries (155). No major TB study has been carried out in this area.

3. Rural Tirupandiyur (BCG trial area)

This rural study area Tirupandiyur is in Tiruvallur district (formerly part of Chingleput district) of Tamilnadu where the major South Indian BCG trial was conducted (Chingleput BCG trial). This study area is about 50 kilometers from Chennai with a total population of about 1500. The main occupation of the people includes agriculture, weaving and small house-hold industries (155). The major BCG efficacy trial conducted by World Health Organization and ICMR in this region revealed 0% efficacy for the vaccine (111).

The population enrolled for the present study (from all the three study area) included both male and female adolescent and adult volunteers. The individuals were enrolled by door-to-door visits and by conducting local field campaigns. Interested individuals/volunteers were only registered for the study. Study objectives and other related information were explained and the written informed consent was obtained from each of these volunteers.

A detailed medical examination along with a questionnaire (Annex-II), which included information on BCG scar status and risk factors associated with tuberculin sensitivity were carried out by a qualified/experienced medical and nursing team. After examination, healthy volunteers who were willing to give consent to tuberculin skin test and blood collection were included for the study.

The individuals were classified into BCG vaccinated and non-BCG vaccinated subjects. The vaccinated group included only subjects with a post vaccine scar, the only sure sign of vaccination. This scar is different from that of small pox; it is smaller in size and is identified by the skin which it covers it, which is smooth, pearly and forms fine folds when squeezed (156). Those with no scar and a definite history of not having received vaccination were grouped as BCG-non vaccinated subjects. Those who gave a history of vaccination but did not have a scar were excluded.

All the recruited volunteers were subjected to tuberculin skin test (Mantoux intradermal test). The test was performed as per the guidelines given by Centre for Disease Control (91).

Tuberculin skin tests were performed by well trained medical laboratory technicians. Proper instructions were orally given to the individuals. 0.1ml of tuberculin PPD 1TU (Span diagnostics, India) and PPD 10TU (Span diagnostics, India) were administrated on each of the forearms of volunteers respectively.

The right and left forearms were randomly allotted to 1TU and 10 TU and the vials were coded, so that the reader did not know the strength of the tuberculin administrated. The transverse diameters of the indurations were measured between 72 and 96 hours and were recorded in millimeters. While reading, erythema was not considered and only the induration was measured.

All the statistical analysis of the present study was carried out in SPSS-Version 14.0. All the descriptive statistics were expressed as mean ± standard deviation and the proportion of tuberculin response with BCG status was analysed using Pearson Chi square. Correlation between the responses of two PPDs and the influence of BCG scar size on tuberculin reaction size were analysed using 2-tailed Pearson's correlation. Logistic regression was used to ascertain the association of risk factors with tuberculin reactivity.

- Results

Baseline characteristics of the study population

A total number of 196 volunteers between the ages of 13 and 34 from the three different study areas were registered between April-2006 and April-2008 for the present study. Twenty three of them were excluded after medical examination (underweight, malnourished, and with chronic infections etc) and their un-willingness to participate in the study. So a total number of 173 healthy subjects were recruited and subjected to tuberculin skin test. The health status of the individuals was examined by the physicians. All the volunteers were healthy and free from HIV, hepatitis and other chronic infections. Forty three percent of the subjects (74/173) were BCG vaccinated as assessed by the presence of BCG scar. Skin testing and reading were done by the same team of experienced health care workers. The summary of the study population for each of the study area is given below and shown in table-5.

Study area 1: Urban Chennai

A total number of 60 volunteers were evaluated for the study. Eight of the volunteers were excluded from the study after medical examination and their un- willingness to participate in the study. So, a total number of 52 young adults with mean age (±SD) of 22.3 ± 3.3 were recruited and subjected to tuberculin skin test. Twenty nine of them were male and 23 of them were female volunteers. Ninety percent of the study subjects were employees of private companies and others were school and college students.

Of them 52 percent (27/52) had a BCG scar. The mean size of BCG scar in this study area was 6.8 mm (range 4 to 15).

Out of 52 percent of subjects with BCG scar, 27 percent were male and 25 percent were female. In the remaining subjects without BCG scar, 29 percent were male (Fig-5).

Study area 2: Sub-urban Pallavaram

A total number of 84 volunteers were evaluated for the study. Nine of the volunteers were excluded from the study after medical examination and their un- willingness to participate in the study. So, a total number of 75 young adults with mean age (±SD) of 19.9 ± 5.3 were recruited and subjected to tuberculin skin test. Forty seven of them were male and 28 of them were female volunteers. Fifty five percent of the study subjects were students and 24 percent of them were employees of private companies.

Of them 44 percent (33/75) had a BCG scar. The mean size of BCG scar in this study area was 8.3 mm (range 4 to 20).

Out of 44 percent of subjects with BCG scar, 28 percent were male and 16 percent were female. In the remaining subjects without BCG scar, 35 percent were male (Fig-6).

Study area 3: Rural BCG trial area

A total number of 52 volunteers were evaluated for the study. Six of the volunteers were excluded from the study after medical examination and their un- willingness to participate in the study. So, a total number of 46 young adults with mean age (±SD) of 19.8 ± 3.1 were recruited and subjected to tuberculin skin test. Twenty two of them were male and 24 of them were female volunteers. Forty percent of the study subjects were employees of private companies and 26 percent of them were students.

Of them 30 percent (14/46) had a BCG scar. The mean size of BCG scar in this study area was 5.9 mm (range 4 to 9).

Out of 30 percent of subjects with BCG scar, 17 percent were male and 13 percent were female. In the remaining subjects without BCG scar, 39 percent were female (Fig-7).

Distribution of scar sizes

In this study the frequency of scar sizes with a diameter of 1-5 mm, 6-10 mm, 11-15 mm and 16-20 mm in all the study areas were compared.

The distribution of scars according to their sizes for each of the study area is shown in fig-8.

More than half of the vaccinated study subjects (58%) have the scar size of 6-10 mm. Thirty percent of the subjects have the scar size of 1-5mm. Only few individuals (12%) have the scar size of above 10 mm. Among the subjects with the scar size of above 10 mm, more subjects were found in sub-urban Pallavaram and no one from the rural BCG trial area.

Base-line characteristics of the study population

Characteristics	Study area			Total
	Urban Chennai	Sub-urban Pallavaram	Rural BCG trial area	
No. of enrolled volunteers	60	84	52	196
No. of withdrawn/ excluded volunteers	8	9	6	23
No. of tuberculin tested and read	52	75	46	173
Gender ratio (M:F)%	56 : 44	63 : 37	48 : 52	57 : 43
Mean age ± SD (in years)	22 ± 3.3	19.9 ± 5.3	19.8 ± 3.1	20.6 ± 4.4
No. with BCG scar	27 (52%)	33 (44%)	14 (30%)	74 (43%)
Mean scar size(mm) ± SD	6.8 ± 2.3	8.3 ± 3.9	5.93 ± 1.5	7.3 ± 3.1

Table – 5. Descriptive summary of the study population. Adolescents and young adults were enrolled. Reasons for Withdrawals/exclusions were unwilling for tuberculin skin test and 'clinically-unfit' declared by physicians. Both the arms were examined for the presence of BCG scar. PPD-1 TU and PPD-10 TU (Span diagnostics, India) were used for tuberculin skin test.

Tuberculin response

A total of 173 study subjects were subjected to tuberculin skin test with PPD-1 TU and PPD-10 TU on each of their forearms. The right and left forearms were randomly allotted to 1 TU and 10 TU as mentioned in the methods. All the indurations were carefully measured individually by two well trained health care workers between 72 and 96 hours. All the tuberculin vials were recoded and the corresponding induration sizes were recorded. All the readings measured individually by two separate readers were the 'same'.

The distribution of reaction sizes to PPD-1TU relative to that of PPD-10U is shown in table-6. While the distribution of PPD-10TU closely follows that of PPD-1TU it is seen that 23 numbers of individuals (Highlighted in the table) were reactive to PPD-1TU but non-reactive to PPD-10TU. These individuals had all had / did not have BCG vaccination. The reasons for this anomaly and relationship with BCG vaccination were unknown. These findings were cannot be explained at this time, and needs to be studied further. This study showed that usage of PPD-10 TU did not reveal any significant detection level as compared to PPD-1 TU. So PPD-1 TU results were alone used in the present study according to the standard guidelines (157).

The mean tuberculin reaction size (± SD) in this study was 8.7 ± 4.9 mm over all. In order to ascertain the frequency and diameters of tuberculin indurations to BCG vaccination, the number and sizes of TST indurations were recorded for the vaccinated and non-vaccinated subjects. The distribution of reaction to PPD-1 TU to those subjects with and without BCG scar is shown in fig-9. For the subjects with BCG scar the mean tuberculin reaction size (± SD) was 9.5 ± 5.5 mm and for the subjects without BCG scar it was 8.2 ± 4.4 mm.

The anti-mode was obtained at 11mm. So subjects with the induration 0-11mm were considered as 'non-reactors' and the subjects with ≥12mm were considered as 'reactors' in this study. This same cut-off point was used in the 7½ and 15 years follow-up studies of Chingleput BCG trial (111, 149).

Table - 6 : Correlation between size of induration to PPD 1 TU and PPD 10 TU for the study subjects

PPD 1TU (mm)	Induration to PPD 10 TU (mm)																								Total
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	21	22	23	24	25	28	
2		2																							2
3	3	2																							5
4	5	5	4	6	3	2		1		1	1	1	2			2									33
5	3	1	1	2	2	2	4	1	2	1				1										1	21
6		1	5		2	1	1																		10
7	1	2	3	1	3		1	2	1			1													15
8	2	1	3	3	3	1	1	1			3	1			2										21
9	2	1			2							1	2												8
10									1		1			1					1						4
11				2	1	1																			2
12			**1**		**1**	**1**	**3**								1	2									11
13					**1**	**2**	**1**		**1**	**1**	1	1	1									1			10
14			**1**		**1**	**1**	**2**	**1**													1		1		8
15	**1**					**1**	**1**					1						1		1					6
16								**1**					1					1							3
17									**1**																1
18																1			1			1			3
19																									
20																1		1	1				1		4
21																	1	1				1			3
22																		2					1		2
25																									1
Total	17	15	18	14	19	12	14	7	6	3	6	6	6	2	3	6	1	6	3	1	1	3	3	1	173

Tuberculin response in urban Chennai

The mean tuberculin reaction size (±SD) in this study area was 8.2 ± 5.0 mm. For the subjects with BCG scar the mean tuberculin reaction size (±SD) was 7.4 ± 5.0 mm and for the subjects without BCG scar it was 9.1 ± 4.8 mm.

Considering 12mm and above to PPD-1TU as 'reactors' the prevalence of reactors and the non-reactors in the subjects (with and without BCG scar) in urban Chennai is shown in fig-10. Totally 16 reactors were found in urban Chennai region and among them 7 subjects had a BCG scar and 9 of them did not. Out of 36 non-reactors, 20 had a scar and 16 of them did not. The proportion of tuberculin response with BCG scar status revealed no statistical significance between tuberculin response and BCG scar status.

The tuberculin responses to PPD-1TU for both the genders (with and without BCG scar) are separately shown in fig-11. Among males, out of 11 reactors, 5 subjects had a scar and 6 of them did not. Out of 18 non-reactors, 9 had a scar and 9 of them did not. There was no statistical significance between tuberculin response and BCG scar status in males.

Among females also, out of 5 reactors, 2 subjects had a scar and 3 of them did not. Out of 18 non-reactors, 11 had a scar and 7 of them did not. There was no statistical significance between tuberculin response and BCG scar status in females.

Tuberculin response in sub-urban Pallavaram

The mean tuberculin reaction size (±SD) in this study area was 9.6 ± 5.3 mm. For the subjects with BCG scar the mean tuberculin reaction size (±SD) was 11.4 ± 5.9 mm and for the subjects without BCG scar it was 8.1 ± 4.3 mm.

The prevalence of reactors and non-reactors in the subjects (with and without BCG scar) in sub-urban Pallavaram is shown in fig-12. Out of 29 reactors, 17 had a BCG scar and 12 of them did not. Out of 46 non-reactors, 16 of them had a scar and 30 of them did not. The proportion of tuberculin response with BCG scar status has revealed a statistical significance ($P < 0.05$).

The tuberculin responses to PPD-1TU for both the genders (with and without BCG scars) are shown in fig-13. Among males, out of 19 reactors, 13 subjects had a scar and 6 of them did not. Out of 28 non-reactors, 8 had a scar and 20 of them did not. This proportion of tuberculin response with BCG scar status in males has revealed a statistical significance ($P < 0.05$).

Among females, out of 10 reactors, 4 subjects had a scar and 6 of them did not. Out of 18 non-reactors, 8 had a scar and 10 of them did not. There was no statistical significance between tuberculin response and BCG scar status in females.

Tuberculin response in rural BCG trial area

The mean tuberculin reaction size (±SD) in this study area was 7.9 ± 4.0 mm. For the subjects with BCG scar the mean tuberculin reaction size (±SD) was 8.7 ± 3.5 mm and for the subjects without BCG scar it was 7.5 ± 4.2 mm.

The prevalence of reactors in the subjects (with and without BCG scar) in rural BCG trial area is shown in fig-14. Out of 8 reactors, 5 of them had a BCG scar and 3 of them did not. Out of 38 non-reactors, 9 of them had a scar and 29 of them did not. The proportion of tuberculin response with BCG scar status in this rural BCG trial area has revealed a statistical significance ($P = 0.05$).

The tuberculin responses to PPD-1TU for both the genders with and without BCG scars are shown in fig-15. Among males, only 2 of them were reactors and both had a scar. Out of 20 non-reactors, 6 had a scar and 14 of them did not. This proportion of tuberculin response with BCG scar status in males has revealed a statistical significance ($P < 0.05$).

Among females, out of 6 reactors, 3 subjects had a scar and 3 of them did not. Out of 18 non-reactors, 3 had a scar and 15 of them did not. There was no statistical significance between tuberculin response and BCG scar status in females.

Influence of BCG scar size on the Tuberculin reaction size

BCG scar is a sensitive indicative marker of the effective BCG vaccination. In this study 74 subjects had a visible scar with mean size (±SD) of 7.38 ± 3.2 mm (range 4 - 20). The mean tuberculin reaction size (±SD) of the BCG vaccinated subjects was 9.5 ± 5.5 mm. The size of tuberculin response according to the BCG scar size is shown in fig-16.

The influence of the size of BCG scar on the size of tuberculin response was studied through Pearson's correlation. The study has showed that the size of scar did not have any influence on the size of tuberculin reaction.

Risk factors associated with tuberculin reactivity

The present study has evaluated the association of certain common risk factors with the tuberculin response by logistic regression analysis using tuberculin response result as the dependent variable. The association was expressed in OR (Odds ratio) with 95% confident intervals.

Out of 173 tested study subjects, 53 of them were reactors. The risk factors included in the questionnaire and their association with the tuberculin reactivity is shown in table-7. The questionnaire included 'The status of BCG scar' as a question to associate BCG scar with tuberculin response. Out of 74 subjects with scar, only 29 of them were reactors (OR 1.7; 95% CI 0.8-3.4). The impact was not statistically significant.

The questionnaire has revealed 12 TB contacts, out of 173 study subjects. Seven of them were tuberculin reactors and five of them were non-reactors (OR 3.4; 95% CI 0.9-12.1). This risk factor of 'Family member (or close contact) with TB' was significantly associated (P=0.05) with the tuberculin reactivity.

Twenty health care workers were included as study subjects who were nurses, clinical lab technicians and other hospital paramedical staff. Among them only six (OR 0.9; 95% CI 0.3-2.8) were reactors. So the risk factor of 'Health care worker' did not have any statistical significance.

All the other risk factors such as prolonged fever, persistent cough etc included in the questionnaire were also not significantly associated with the tuberculin response in the present study.

Risk factors associated with tuberculin reactivity

Risk factor	No. of subjects			Unadjusted OR (95% CI)
	TST non-reactors (n=120)	TST reactors (n=53)	Total (n=173)	
Presence of BCG scar	45 (37.5)	29 (54.7)	74 (42.7)	1.7 (0.8-3.4)
Family member (close contact) with TB	5 (4.1)	7 (13.2)	12 (6.9)	3.4 (0.9-12.1)*
Residence in a refugee camp	0 (0)	0 (0)	0 (0)	NA (NA)
Residence in a slum	0 (0)	0 (0)	0 (0)	NA (NA)
Health care worker	14 (11.6)	6 (11.3)	20 (11.5)	0.9 (0.3-2.8)
Asthma	3 (2.5)	2 (3.7)	5 (2.8)	1.7 (0.1-16.5)
Smoking, Alcohol & tobacco use	9 (7.5)	9 (16.9)	18 (10.4)	2.4 (0.8-6.9)
Prolonged Fever	8 (6.6)	7 (13.2)	15 (8.6)	1.7 (0.5-5.5)
Persistent Cough	17 (14.1)	11 (20.7)	28 (16.1)	1.8 (0.7-4.3)
Wheezing	3 (2.5)	1 (1.8)	4 (2.3)	0.7 (0.04-11.8)
Fatigue	0 (0)	0 (0)	0 (0)	NA (NA)
Lack of appetite	0 (0)	0 (0)	0 (0)	NA (NA)
Sudden weight loss	4 (3.3)	1 (1.8)	5 (2.8)	0.5 (0.06-5.1)

Table - 7. Risk factors associated with tuberculin reactivity. Figures in the parenthesis represent the percentage of the total.
NA - Not applicable.
* - Statistically significant (P = 0.05)

GENDER AND BCG SCAR STATUS IN URBAN CHENNAI

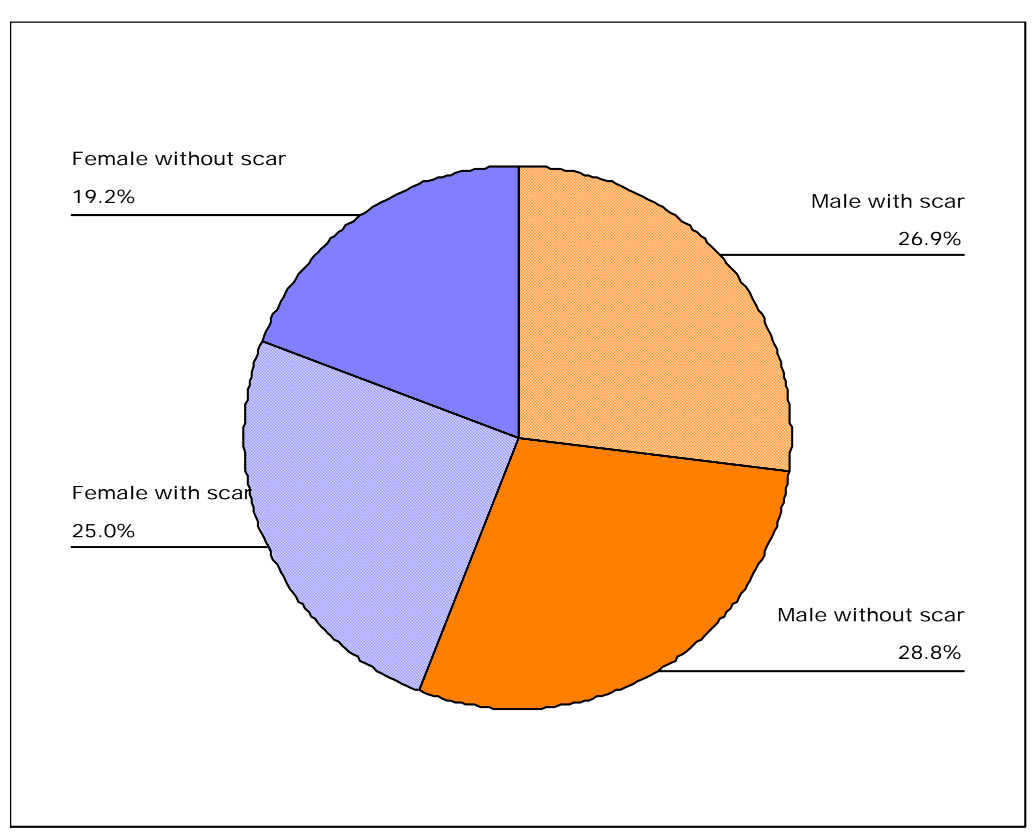


Fig - 5. BCG scar status according to gender in urban Chennai study subjects.

GENDER AND BCG SCAR STATUS IN SUB-URBAN PALLAVARAM

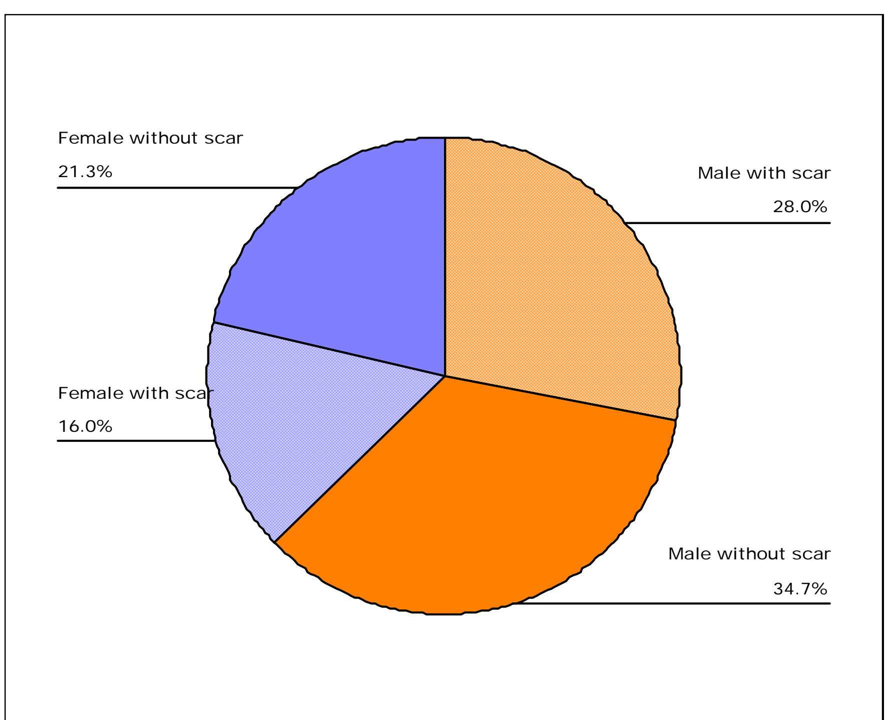


Fig - 6. BCG scar status according to gender in sub-urban Pallavaram study subjects.

GENDER AND BCG SCAR STATUS IN RURAL BCG TRIAL AREA

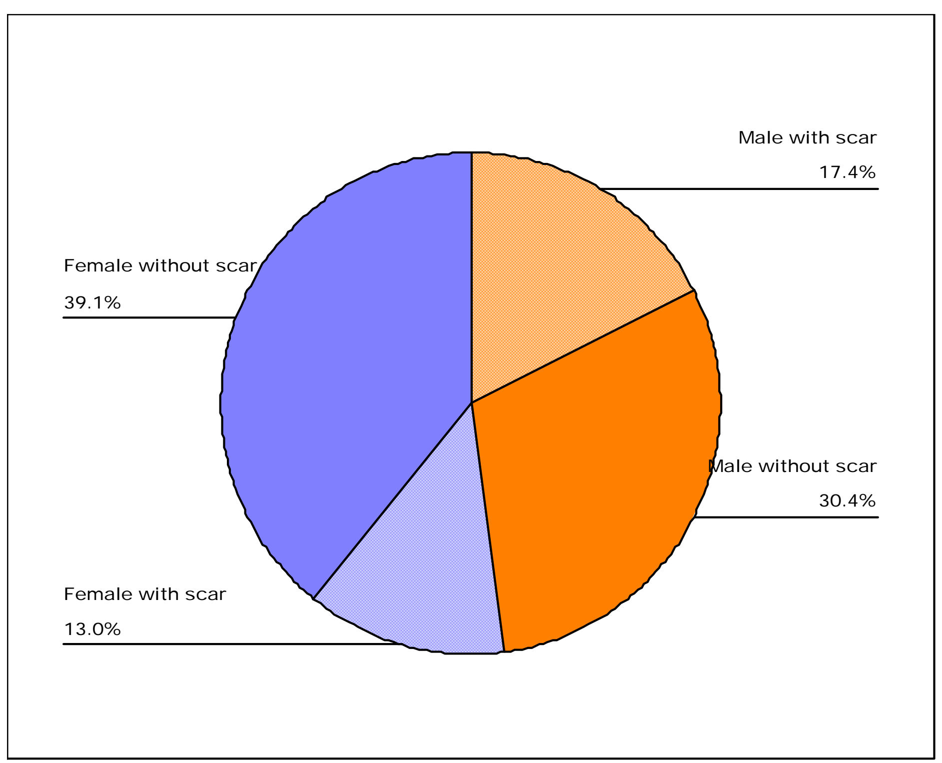


Fig - 7. BCG scar status according to gender in rural BCG trial area study subjects.

BCG SCAR SIZES IN THE STUDY AREAS

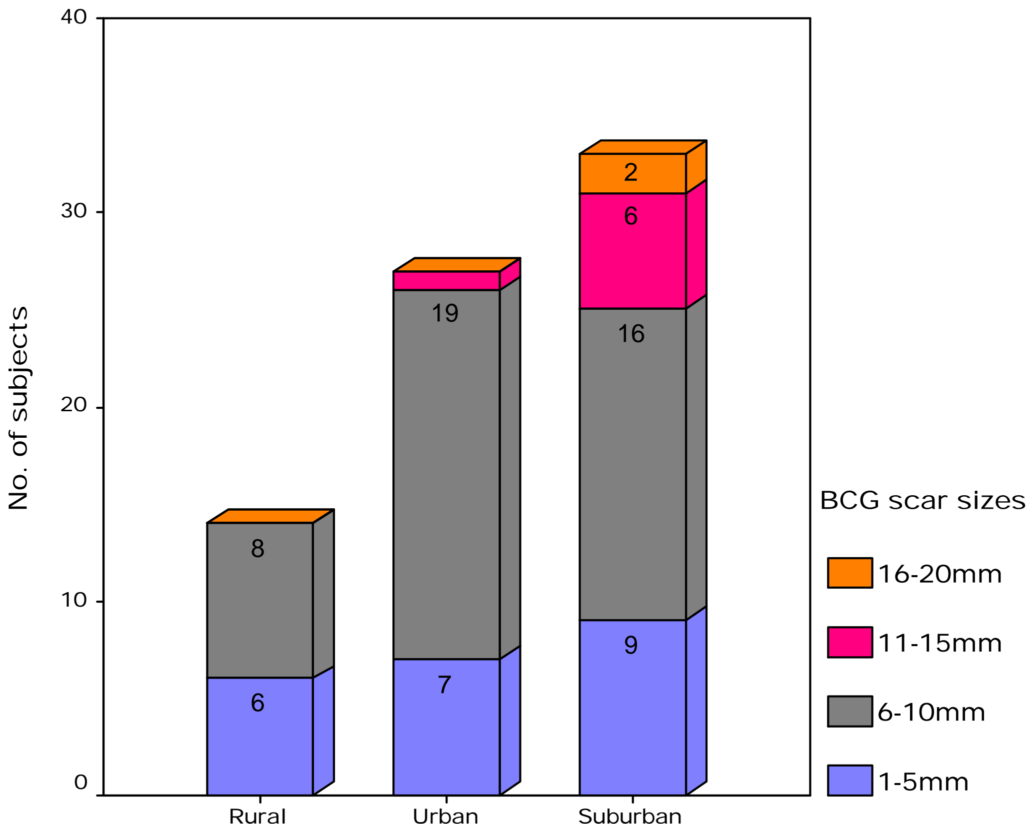


Fig - 8. **Distribution of BCG scar sizes in the study areas.** More than half of the study subjects had the scar size of 6-10mm. Only few study subjects had the scar size of above 10mm. Present study has revealed that no rural subjects were found with scar size of >10mm.

Figures in the bars represent the number of subjects.

DISTRIBUTION OF PPD-1TU RESPONSE

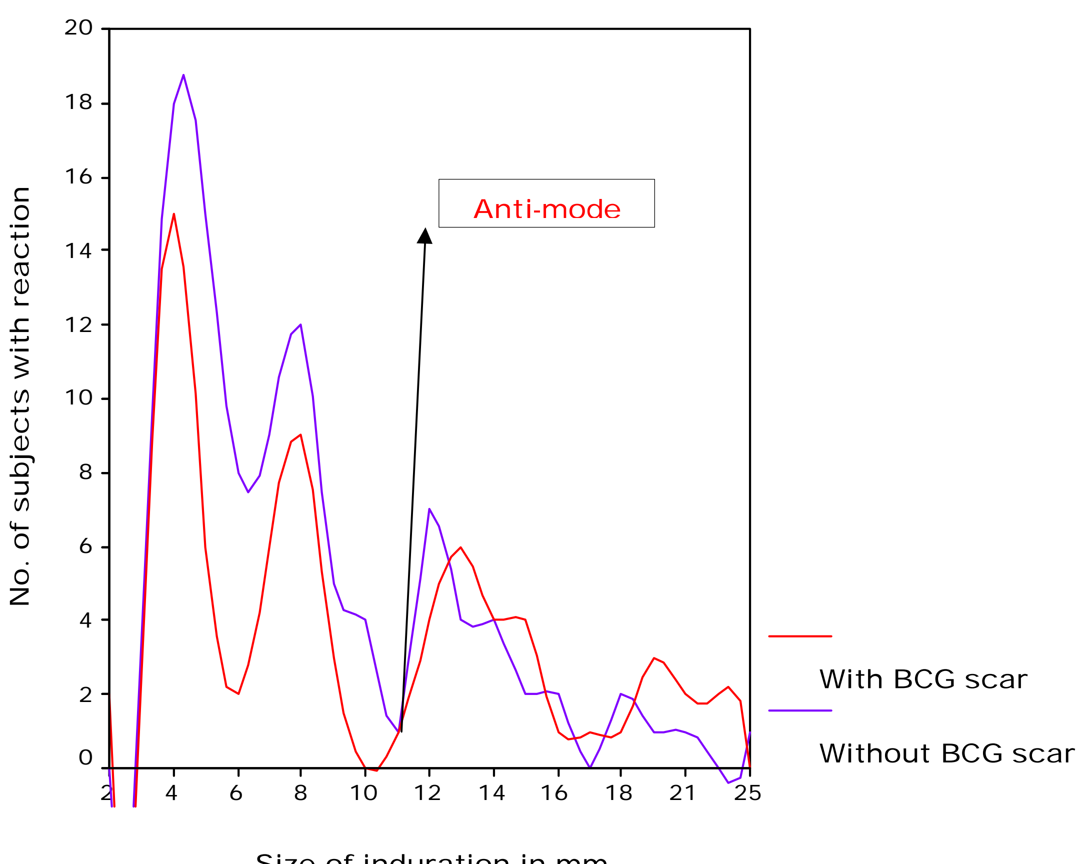


Fig – 9. Distribution of PPD-1TU response according to induration size in subjects with and without BCG scar. Tuberculin skin test indurations were recorded between 72 and 96 hours for both the vaccinated (n=74) and unvaccinated subjects (n=99). The anti-mode was obtained at 11mm. So, subjects with the induration size of 0-11mm were considered as 'non-reactors' and ≥12mm were considered as 'reactors' in this study.

TUBERCULIN RESPONSE IN URBAN CHENNAI

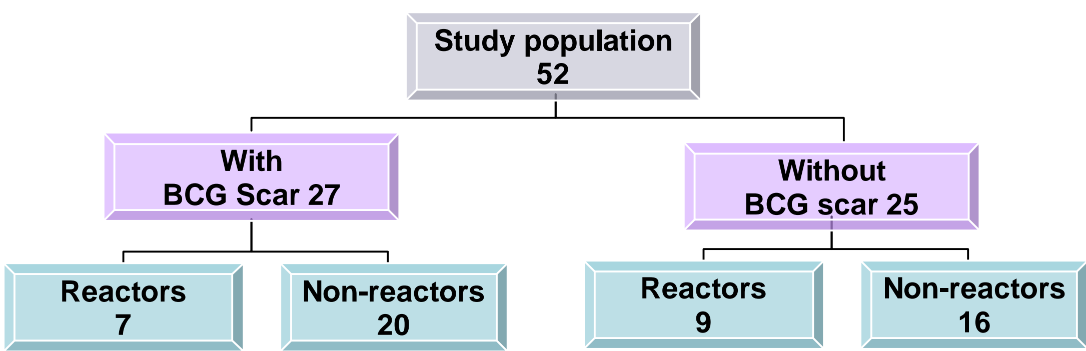
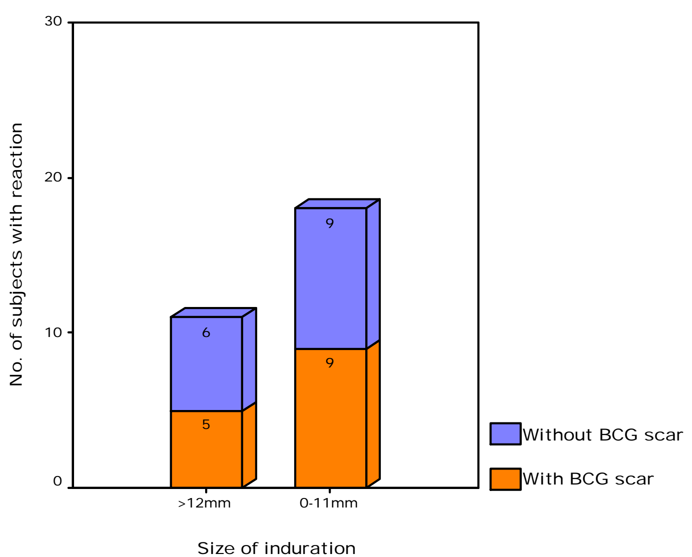


Fig - 10. Tuberculin responses according to BCG scar status in urban Chennai study population. Tuberculin response to PPD-1 TU was classified based on the size of induration, 0-11mm as 'non-reactors' and ≥12mm as 'reactors'. The proportion of tuberculin response with BCG scar status was not statistically significant.

(a) TUBERCULIN RESPONSE AMONG MALES



(b) TUBERCULIN RESPONSE AMONG FEMALES

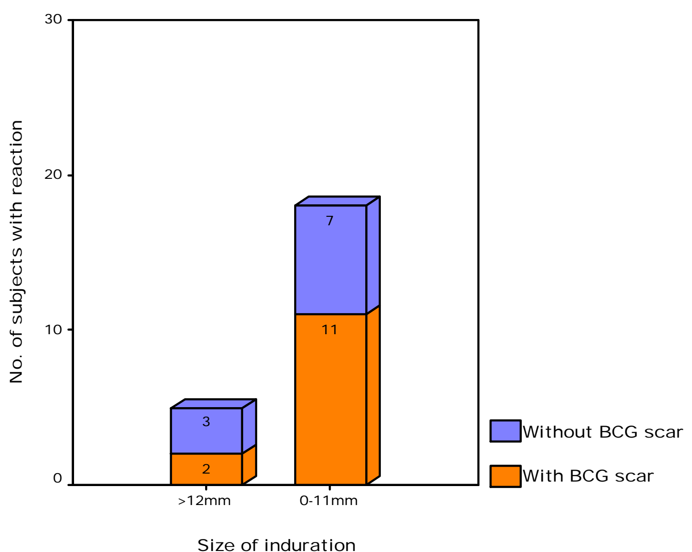


Fig – 11. Tuberculin responses according to BCG scar status among urban Chennai study subjects. The proportion of tuberculin response with BCG scar status among males and females was not statistically significant.
Figures in the bars represent the number of subjects.

TUBERCULIN RESPONSE IN SUB-URBAN PALLAVARAM

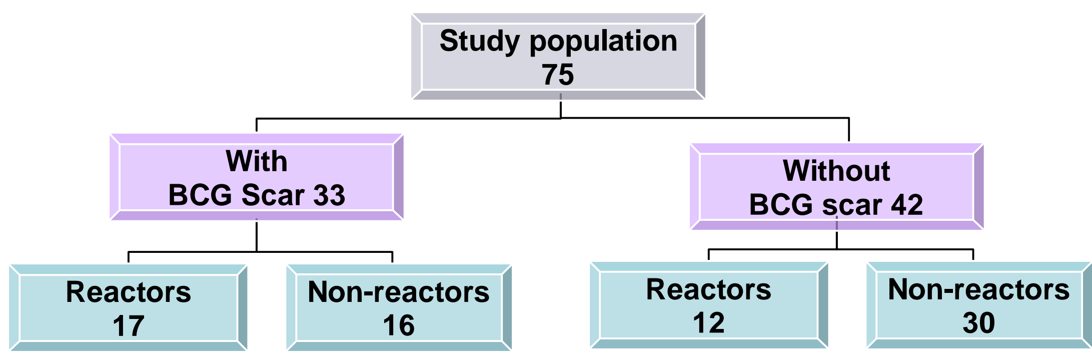
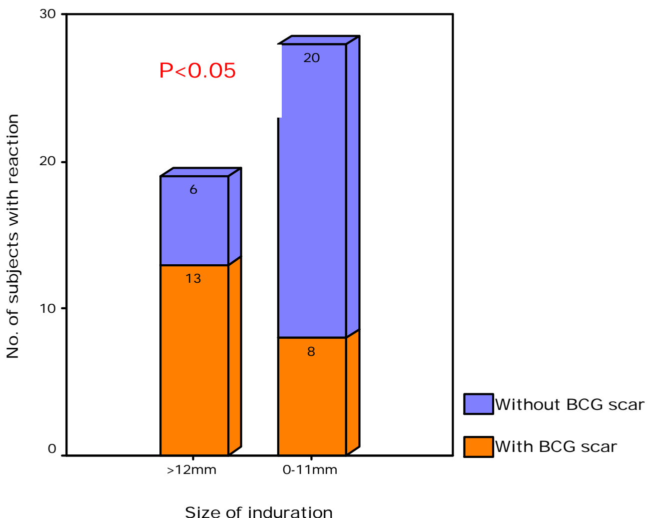


Fig -12 . Tuberculin responses according to BCG scar status in sub-urban Pallavaram study population. Tuberculin response to PPD-1 TU was classified based on the size of induration, 0-11mm as 'non-reactors' and ≥12mm as 'reactors'. The proportion of tuberculin response with BCG scar status has revealed a statistical significance **($P < 0.05$)**.

(a) TUBERCULIN RESPONSE AMONG MALES



(b) TUBERCULIN RESPONSE AMONG FEMALES

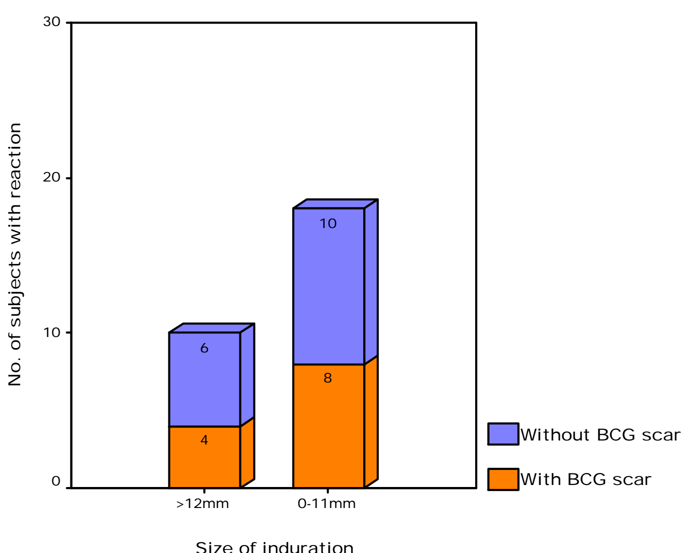


Fig –13. Tuberculin responses according to BCG scar status among sub-urban Pallavaram study subjects.

(a) The proportion of tuberculin response with BCG scar status among males has revealed a statistical significance **($P < 0.05$)**.

(b) The proportion of tuberculin response with BCG scar status among females was not statistically significant.

Figures in the bars represent the number of subjects.

TUBERCULIN RESPONSE IN RURAL BCG TRIAL AREA

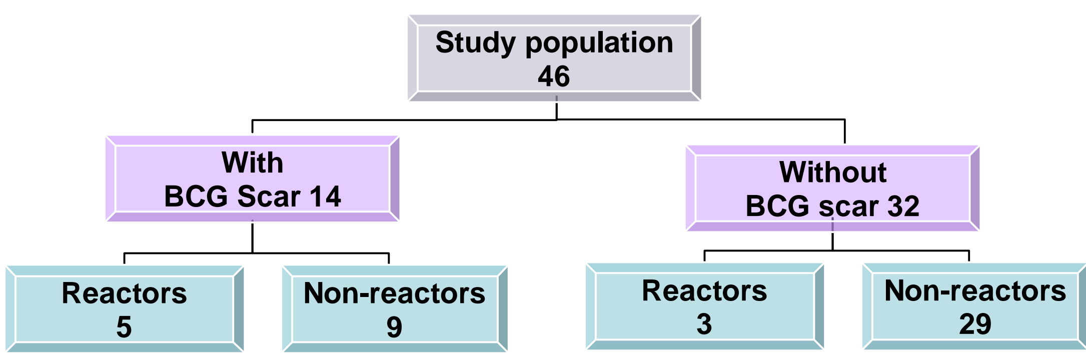
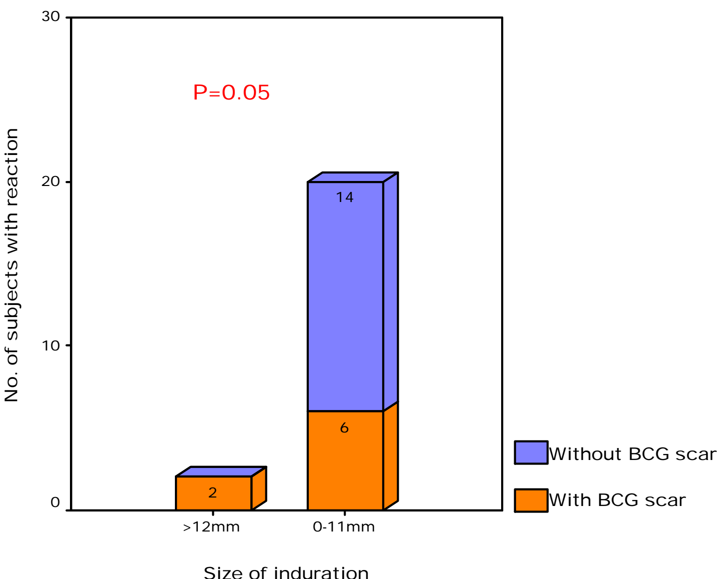


Fig -14. Tuberculin responses according to BCG scar status in rural BCG trial area study population. Tuberculin response to PPD-1 TU was classified based on the size of induration, 0mm-11mm as 'non-reactors' and ≥12mm as 'reactors'. The proportion of tuberculin response with BCG scar status has revealed a statistical significance.
($P < 0.05$).

(a) TUBERCULIN RESPONSE AMONG MALES



(b) TUBERCULIN RESPONSE AMONG FEMALES

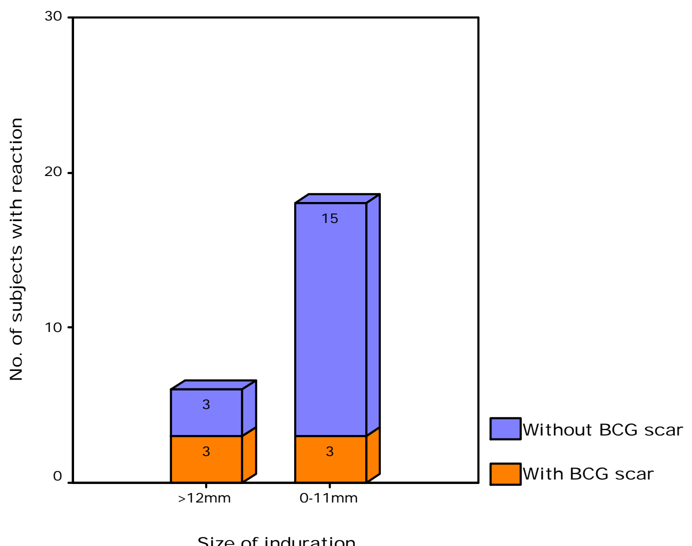


Fig – 15. Tuberculin responses according to BCG scar status among rural BCG trial area study subjects.

(a) The proportion of tuberculin response with BCG scar status among males has revealed a statistical significance **(*P* = 0.05)**.

(b) The proportion of tuberculin response with BCG scar status among females was not statistically significant.

Figures in the bars represent the number of subjects.

TUBERCULIN RESPONSE ACCORDING TO BCG SCAR SIZE

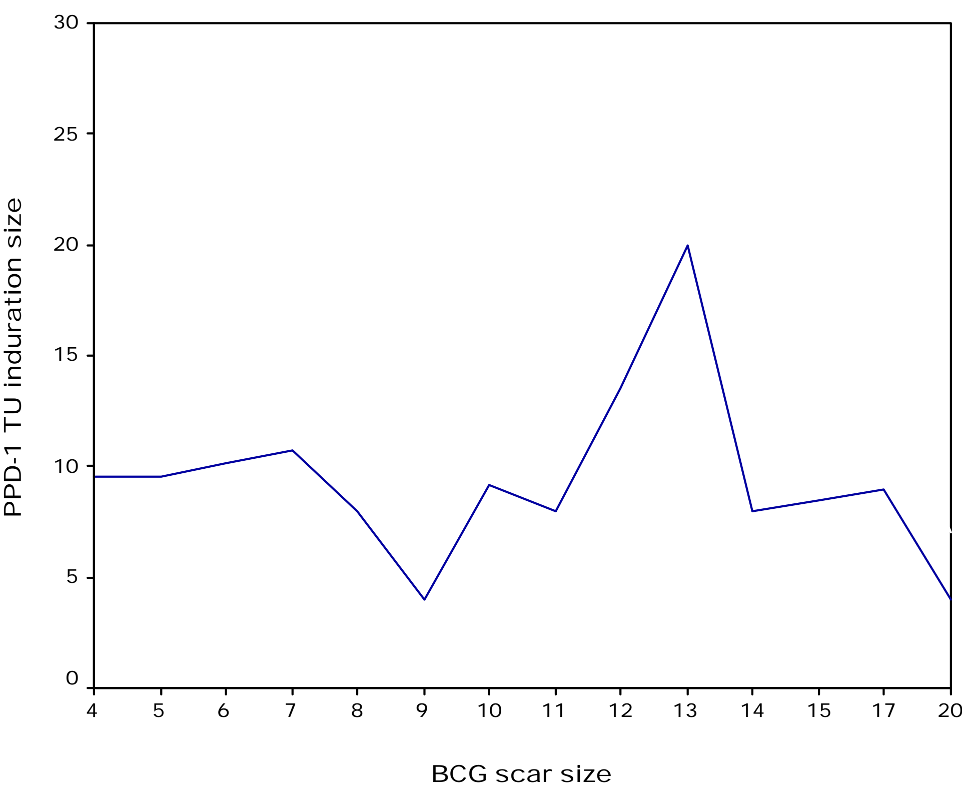


Fig – 16. Tuberculin response according to BCG scar size in the study subjects (n=74). The size of scar did not have any influence on the size of tuberculin reaction.

Distribution of BCG scar

- Out of 173 adolescent and young adult-volunteers registered from the three different study areas, 43% of the subjects (74/173) were BCG vaccinated as assessed by the presence of BCG scar.
- In urban Chennai 52 percent had BCG scar. The mean size of BCG scar in this study area was 6.8mm (range 4 to 15).
- In sub-urban Pallavaram 44 percent had BCG scar. The mean size of BCG scar in this study area was 8.3mm (range 4 to 20).
- In rural BCG trial area (Tirupandiyur) 30 percent had BCG scar. The mean size of BCG scar in this study area was 5.9mm (range 4 to 9).
- More than half of the vaccinated study subjects (58%) had the scar size of 6-10mm.
- The distribution of scar sizes did not show any significant difference according to gender in all the three study area.

Tuberculin response

- This study showed that there is no significant difference between the induration sizes of PPD-1 TU and PPD-10 TU. Hence, it is clear that the detection level of PPD-10 TU was not superior when compared to PPD-1 TU.

- The mean tuberculin reaction size (± SD) to PPD 1TU in this study was 8.7 ± 4.9 mm over all. For the subjects with BCG scar the mean tuberculin reaction size (± SD) was 9.5 ± 5.5 mm and for the subjects without BCG scar it was 8.2 ± 4.4 mm.

Influence of BCG scar on Tuberculin response in urban Chennai

- In urban Chennai, present study has revealed no statistical significance between tuberculin response and BCG scar status for both male and female.

- In sub-urban Pallavaram, the proportion of tuberculin response with BCG scar status has revealed a statistical significance ($P < 0.05$). This significance was seen among males and not in females.

- In rural BCG trial area (Tirupandiyur) the proportion of tuberculin response with BCG scar status has revealed a statistical significance ($P = 0.05$). In this area also the significance was seen among males not in females.

Influence of BCG scar size on the Tuberculin reaction size

- In this study population, the size of scar did not have any influence on the size of tuberculin reaction.

Risk factors associated with tuberculin reactivity

- This risk factor of 'Family member (or close contact) with TB' was significantly associated (P=0.05) with the tuberculin reactivity. All the other risk factors included in the questionnaire were not significantly associated with the tuberculin reactivity in the present study.

- Chapter – **II** : Standardization of Experiments

The time duration required for transporting the blood samples from field study areas to immunology cell culture laboratories may cause loss in the viability and functional activity of immune cells. At the same time, it is very difficult to bring all those volunteers to the laboratories as it is expensive and time consuming. Besides, it will result in loss of wages for the subjects. Because of these difficulties, immuno-epidemiological studies are very limited in the field of tuberculosis. Hence it becomes very important to document the loss of viability of the cells at room temperature with the elapse of time after blood collection.

In the present study, the first part of the laboratory experiment was designed to determine the loss of viable PBMC at different time points from the time of blood collection that are required to transport the blood samples from study area to the cell culture laboratory.

Blood samples were collected from apparently healthy volunteers (These subjects were not included in the study and utilized for this standardization experiment alone). About 30 ml of peripheral blood was collected by venupuncture in heparinised vacutainer tubes (BD, USA) and stored at room temperature. Five ml of blood was collected separately for serum. Peripheral blood mononuclear cells (PBMC) were isolated from the stored blood at different time points; immediately after collection (0 hr), 1hr, 2hr, 3hr, 4hr and at 6 hours from collection. At each time point, blood was aseptically layered on equal volume of Histopaque (Sigma Aldrich, USA) and centrifuged at 400g for 30 min. The supernatant (plasma) was removed and the peripheral blood mononuclear cells (PBMC) were separated in the inter-

phase layer by density gradient, washed three times with RPMI-1640 medium (Gibco, USA) and resuspended in RPMI 1640 containing 10% autologous serum.

Twenty microliters of the re-suspended PBMCs isolated at different time points from stored blood were diluted in 160µl RPMI-1640 and stained with 20µl 0.4% Trypan blue (Gibco, USA). After 5 minutes of incubation at room temperature, the cell suspensions were placed in Neubaur counting chamber (Feinoptik, Germany) and the cells were counted.

Both stained and unstained cells in the four corner squares were counted to enumerate the count using the formula;

$$\text{Total PBMC count} = \frac{\text{No. of cells counted in the corner squares}}{4} \times \text{Dilution factor} \times 10^4$$

Similarly, the percentage of viable peripheral blood mononuclear cells was also assessed. The percentage of cell viability was calculated using the formula;

$$\text{Cell viability (\%)} = \frac{\text{Total number of unstained viable cells}}{\text{Total number of cells (stained and unstained)}} \times 100$$

THE LOSS OF VIABLE PBMC

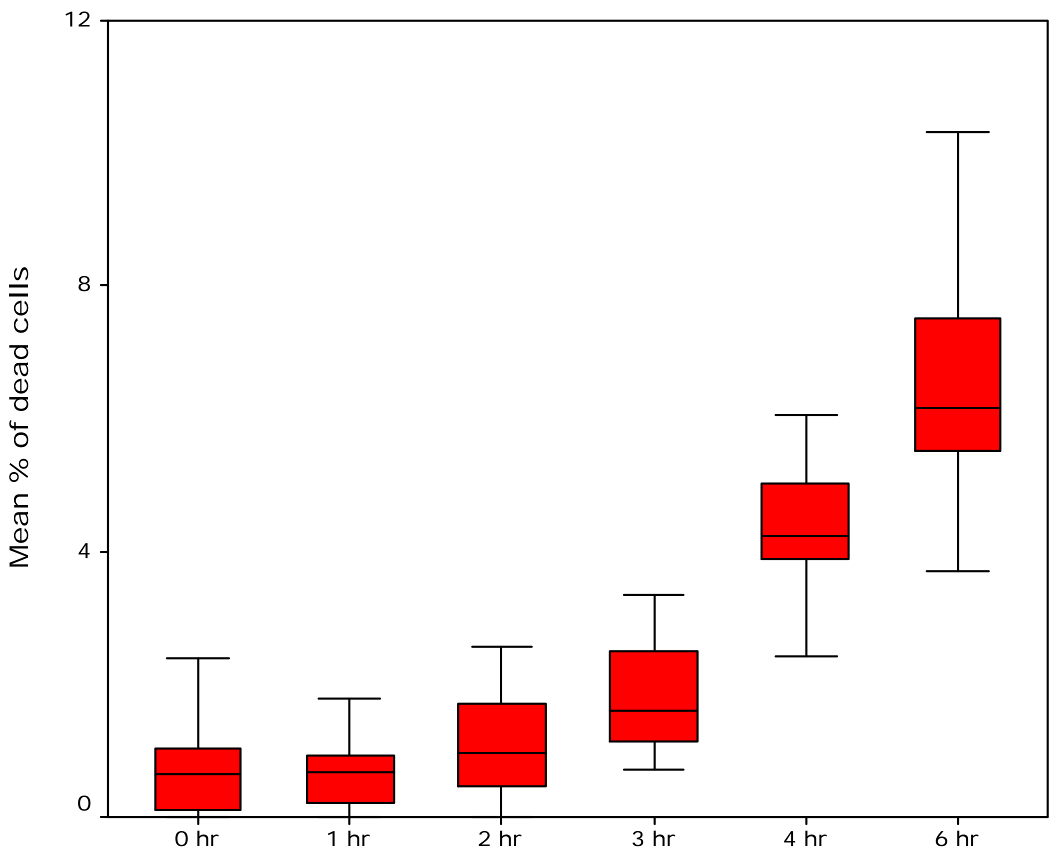


Fig - 17. The loss of viable PBMC at different time points. Blood samples (n=20) were examined at different time points from blood collection for viable PBMC. Even after 6 hours, only 6% of the cells were dead. Hence, blood can be transported to the lab within six hours to get reliable results.

The loss of viable PBMC

The loss of viable PBMC at six different time points from blood collection at room temperature was performed by using trypan blue exclusion method. A total number of twenty blood samples were collected from different volunteers.

At each time point, 5 to 6 ml of blood was taken for the isolation of cells. The mean percentage of dead cells at each time point was obtained as described in methods. The gradual increase of dead cells along with time point is shown in fig-17.

The mean percentage of dead peripheral blood mononuclear cells for 0-hour was 0.64%. The next three time points from blood collection (one hour, 2 hours and 3 hours) showed 0.74%, 1.08% and 1.78% of dead cells respectively. But the last two time points (4 hours and 5 hours) from blood collection showed 4.36% and 6.49% of dead PBMC.

The present experiment has suggested that even after six hours from blood collection, the viability of PBMC was more than 90% at room temperature.

Processing and quantification of *M. tuberculosis* - H37Rv

Stock cultures of *M. tuberculosis* H37Rv strain were obtained from the Bacteriology department of Tuberculosis Research Centre, Chennai, South India. The H37Rv strains have been carefully maintained in the department and periodically evaluated for viable counts according to a strict protocol followed by the Tuberculosis Research Centre and adopted internationally by supranational laboratories.

M. tuberculosis - H37Rv grown in a LJ slant media was obtained and the rough raised colonies were carefully scrapped using a sterile inoculation loop and emulsified with sterile distilled water using a cyclomixer. This suspension was then subcultured in Middlebrook 7H9 broth (BD, USA) and incubated at 37^0C for 6 days to get log phase culture.

A total number of 20 subcultures were made to use throughout the study in order to use a single batch of organisms. All the subcultures were labeled and stored at -80^0C for *in vitro* experimental infections.

During the experiment schedules, the cultures were thawed and to minimize clumping and for accurate quantification, the cultures were vortexed with sterile autoclaved glass beads for 5 minutes and centrifuged at 2000 rpm for 10 minutes. Bacteria remaining in the supernatant were used for infections. The total number of bacilli in the suspension was ascertained by counting in Thoma counting chamber (Brand, UK) using the formula;

$$\text{Bacilli per microlitre} = \frac{\text{Counted bacilli}}{0.2 \text{ X } 0.1 \text{ X } 1 \text{ / Dilution factor}}$$

Where;

0.2 = Counted surface in mm^2

0.1 = Depth

The viability of bacilli was enumerated by CFU counts on Middlebrook 7H11 agar plates supplemented with OADC (Oleic Acid Dextrose Complex) (BD, USA). The bacilli suspension was diluted into 1:10, 1:100, 1:1000 with sterile distilled water and 25 microlitre of each dilution was separately seeded on Middlebrook 7H11 agar plates supplemented with OADC. The inoculated plates were incubated at 37^0C for 2-3 weeks to get visible colonies. All the colonies were carefully counted and the number of viable bacilli (CFU) was calculated by using the formula;

Number of viable bacilli per ml = Number of colonies x 40 x Dilution factor

Where;

40 = Multiplying factor to convert 25μl to 1 ml

Quantification of *M. tuberculosis* H37Rv

Twenty subcultures that were obtained from single mother culture were stored at -80^0C and were thawed / processed only during the experiments. The processed single cell bacilli were counted in Thoma cell counter and the viability of bacilli was enumerated by performing colony forming units (CFU).

Twelve of the twenty subcultures were utilized for this present study. The total number of bacilli revealed by Thoma count and its viable number is shown in table - 8. In general, after counting the bacilli with Thoma cell counter the concentration of bacilli was adjusted to 2 $X10^6$ bacilli with RPMI 1640 medium for practical convenience.

This experiment revealed 40 - 80% of viable bacilli from its total bacterial count revealed by Thoma cell counter. The total number of bacilli and its viable count were separately labeled and used according to the experiments.

Quantification of *M. tuberculosis* H37Rv

Subculture	Thoma cell count (per ml)	Viable count (per ml)
Batch 1	2 X 10^6 bacilli	16 X 10^5 cfu
Batch 2	2 X 10^6 bacilli	10 X 10^5 cfu
Batch 3	2 X 10^6 bacilli	12 X 10^5 cfu
Batch 4	2 X 10^6 bacilli	8 X 10^5 cfu
Batch 5	2 X 10^6 bacilli	12 X 10^5 cfu
Batch 6	2 X 10^6 bacilli	10 X 10^5 cfu
Batch 7	2 X 10^6 bacilli	10 X 10^5 cfu
Batch 8	2 X 10^6 bacilli	8 X 10^5 cfu
Batch 9	2 X 10^6 bacilli	12 X 10^5 cfu
Batch 10	2 X 10^6 bacilli	14 X 10^5 cfu
Batch 11	2 X 10^6 bacilli	14 X 10^5 cfu
Batch 12	2 X 10^6 bacilli	10 X 10^5 cfu

Table - 8. **Enumeration of bacterial count and viable CFU.** All the subculture batches were derived from a single mother culture of *M. tuberculosis* H37Rv obtained from the Bacteriology Department, Tuberculosis Research Centre. At each time, the Thoma cell count was adjusted to 2 X 10^6 bacilli with RPMI-1640 for practical convenience. Viable count was performed by using Middlebrook 7H11 agar medium.

All the results of recruited volunteers who were subjected to tuberculin skin test with PPD-1TU and PPD-10TU were analyzed and only healthy volunteers in the age group of 15-24 who were willing to give blood were included for the immunological laboratory experiments. Volunteers who were underweight, malnourished, chronic infections and with the habits of cigarette smoking, alcoholism and tobacco-use were not included for the immunological laboratory experiments.

All the included study subjects for immunological laboratory experiments were grouped into four natural study groups as follows.

Group	Description	Referred in this study as
1	With BCG scar; tuberculin reactors	Vaccinated reactors
2	With BCG scar; tuberculin non-reactors	Vaccinated non-reactors
3	Without BCG scar; tuberculin reactors	Non-vaccinated reactors
4	Without BCG scar; tuberculin non-reactors	Non-vaccinated Non-reactors

- Chapter – III : Immunological laboratory work

To determine the anti-mycobacterial activity of monocytes and monocyte derived macrophages, peripheral blood mononuclear cells (PBMC) were cultured in a 24 well flat bottom tissue culture plates (Greiner, Germany).

PBMCs were cultured in tissue culture plates at $2.5X10^6$ PBMC per well. To measure the phagocytic index, a part of PBMCs with same concentration were separately layered on sterile coverslips that were placed within the wells of a 24 well flat bottom tissue culture plates (Greiner, Germany). Plates were incubated at 37°C under 5% CO_2 (Kendro, Germany). Three hours after incubation, the non-adherent lymphocytes were washed out by gentle aspiration. Monocytes and macrophages are adherent cells that will remain in the culture plate. Fresh RPMI-1640 medium was added to the adherent monocytes and the cells were observed under phase contrast microscope (Nikon, Japan) everyday to check for contamination.

24 hours post culture (Day-1) adherent cells were treated as monocytes. On day-3, once again the cells were washed with RPMI-1640 medium and fresh medium with 10% autologous serum was added and the cells were retained in the incubator for 6 days allowing the monocytes to mature into macrophages.

The viability of the adhered macrophages was determined on 6th day. Ice cold PBS was added to one of the well and after 15 minutes cells were scrapped by a cell scraper and the viability of macrophages was determined once again by trypan blue exclusion method.

To measure phagocytosis, macrophages layered on coverslips were infected with *M. tuberculosis*-H37Rv at multiplicity of infection of 10 bacilli:1 macrophage ratio. Infected macrophages were incubated for 3 hours at 37°C under 5% CO_2 to allow for phagocytosis.

After 3 hours the supernatants were gently aspirated and washed with serum free RPMI-1640 to remove unengulfed bacilli. Coverslips were treated with 1% formalin and stained with Kinyoun's acid fast staining method to reveal the bacilli-engulfed macrophages. Stained coverslips were mounted on slides using DPX and observed under microscope. Each time 100 fields were examined for counting.

Phagocytic index was calculated using the formula;

$$\text{Phagocytic Index} = \frac{\text{No. of bacilli-engulfed macrophages}}{\text{Total no. of macrophages}} \times 100$$

Principle of the Assay

This method utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo(c) fluorene (R1). This R1 reagent undergoes alkaline autooxidation, which is accelerated by superoxide dismutase (SOD). Autooxidation of R1 yields a chromophore which absorbs maximally at 525nm.

This method also utilizes a second proprietary reagent 1-methyl-2-vinylpyridinium (R2) that eliminates major interferences normally caused by mercaptans (RSH), such as glutathione, in the sample. Reagent R2 traps mercaptans by means of a rapid alkylation reaction.

Unit definition

The SOD activity is determined from the ratio of the autooxidation rates measured in the presence (Vs) and in the absence (Vc) of SOD. One SOD-525 activity unit is defined as the activity that doubles the autooxidation rate of the control blank (Vs/Vc=2).

Assay for samples

The monocytes/partially matured macrophages were infected with *M. tuberculosis* H37Rv at the multiplicity of infection (MOI) of 10:1 (Ten bacilli to one monocyte) and the plates were incubated at 37°C under 5% CO_2 (Kendro, Germany). Three hours after the infection, the supernatant was aseptically collected and centrifuged at 10,000g for 10 minutes to pellet out the bacilli. The supernatant was then subjected to superoxide dismutase assay. Supernatant from uninfected cells of the same subjects were used as the controls.

Assay method

- The spectrophotometer (Varian, Australia) was adjusted to zero at 525±2 nm with deionized water.
- 900 μl buffer (2 – amino-2-methyl-1,3-propanediol) was added to a test tube for each blank or sample.
- 40 μl of deionized water (as blank) and 40 μl of sample were added to the respective tubes.
- 30 μl of the R2 solution was added to the test tubes and vortexed.
- Both blank and sample test tubes were incubated at 37^0C for 1 minute.
- After incubation 30 μl of the R1 solution was added to the test tubes and vortexed gently.
- Test tube mixtures were immediately transferred to spectrophotometer cuvette and the absorbance was measured at 525nm.

Vs/Vc Ratio

- An average reading of four blank rate determinations was used for Vc.
- Each sample rate (Vs) was divided by the average rate of blank samples.

Determining SOD activity

The SOD activity in SOD-525 units was derived from the equation;

$$\frac{Vs}{Vc} = 1 + \frac{(SOD)}{\alpha\,(SOD) + \beta}$$

By rearranging the equation;

$$SOD = \frac{0.93\,(Vs/Vc - 1)}{1.073 - 0.073\,(Vs/Vc)}$$

Where,

Vs = Rate of sample containing SOD

Vc = Average rate of blank (without SOD) samples (Mean of 4 assays)

SOD = SOD activity of the sample in SOD-525 units

α = 0.073 (Dimensionless coefficient)

β = 0.93 (Coefficient in SOD-525 units)

Glutathione assay

Principle of the Assay

Principle is based on a chemical reaction which proceeds in two steps. The first step leads to the formation of substitution products (thioethers) between a reagent, R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate), and all mercaptans (RSH) which are present in the sample.

Cl
RSH
SR
F_3C
N
Reagent R1
Thioethers

The second step is an elimination reaction that takes place under alkaline conditions. This reaction is mediated by reagent R2 (30% NaOH) which specifically transforms the substitution product obtained with glutathione into a chromophoricthione with a maximal absorbance at 400 nm.

GS
pH > 13
S
F_3C
N+
N
GSH adduct
Thione

Assay for Standard

Before each new series of assays, a standard curve was prepared with five different concentrations of glutathione. These five concentrations covered

the range of 20-100 µmol/L in the reaction medium (spectrophotometric cuvette). Metaphosphoric acid working solution was prepared by dissolving 5 grams metaphosphoric acid in 100 ml of water. 0.5 mMol/l of glutathione working standard solution was prepared by dissolving 15.36 mg of Glutathione into 100 ml of 5% metaphosphoric acid working solution. From this solution 40ul, 80ul, 120ul, 160ul and 200ul were aliquotted and it was finalized to a concentration of 900ul with buffer. This bought the glutathione concentration of 20, 40, 60, 80 and 100 mMol per liter. To all the test tubes 50 µl of solution R1 was added and thoroughly mixed followed by the addition of 50 µl of solution R2 and thoroughly mixed. Test tubes were incubated at 25°C for 10 min in the dark. The final absorbances (A) were measured at 400 nm.

Assay for Samples

The cultured macrophages were infected with *M. tuberculosis* H37Rv at multiplicity of infection 10:1 and the culture plates were incubated at 37°C under 5% CO_2 (Kendro, Germany). The bacilli were allowed for phagocytosis for 2 hours. After the incubation time the extracellular unphagocytosed bacilli were washed and fresh medium was added.

Three days after the infection the infected macrophages were detached from the culture plates using a cell scrapper. The cell suspension was pelletted by centrifuging at 10,000g for 10 minutes at 4^0C. An equal volume of ice cold 10% metaphosphoric acid was added to the pellet and centrifuged at 10,000g at 4^0C for 8 minutes. The supernatant was collected and used for glutathione estimation.

Assay method

- The spectrophotometer (Varian, Australia) absorbance was adjusted to zero at 400 nm with buffer.
- Three independent measurements of blank absorbance (A_0) were performed at 400 nm.
- The blanks were measured only after 10 min of incubation at room temperature.
- 300µl of sample was taken and the volume was brought to 900 µl with buffer.
- 50 µl of solution R1 was added and thoroughly mixed.
- Then 50 µl of solution R2 was added and thoroughly mixed.
- Test tubes were incubated at 25 °C for 10 min in the dark.
- The final absorbances (A) were measured at 400 nm.

Calculation of results

GSH Concentration was calculated from the following equation;

$$GSH = \{(A-A_0) / (E \times l)\} \times D$$

Where;

GSH is the initial glutathione concentration in the sample, expressed as molar concentration.

A and A_0 are the absorbances measured in the presence and in the absence of sample, respectively.

E is the apparent molar extinction coefficient of the product measured at 400 nm.

l is the optical path (cm).

D is the dilution factor of the sample.

Whole blood assay involves the culture of heparinised blood. These cultures contain not only the peripheral blood mononuclear cells but also red cells, leukocytes and platelets. So this method additionally allows full interplay of cellular and humoral factors.

Method

Heparinised blood was diluted into1 in 3 concentration with serum-free RPMI-1640 medium and cultured in a 24 well culture plates at 1ml per well and immediately followed by the *in vitro* infection of *M. tuberculosis* H37Rv at 10^5 CFU per well. Plates were incubated at 37°C under 5% CO_2 (Kendro, Germany). After 24 hours and 96 hours of infection, the samples were aseptically transferred and centrifuged at 10000g for 10 minutes. The supernatants were collected and stored at – 80^0C for cytokines estimation. The 24 hour supernatant samples were used for TNF- α estimation and the 96 hour supernatant samples were used for IFN- γ estimation.

Capture antibody preparation

The capture antibody was added at 1:250 dilution to the coating buffer.

Working Detector Preparation

Detection antibody and enzyme reagent were added at 1:250 dilution to assay diluent and vortexed well. Working detector was prepared within 15 minutes prior to the addition.

Standards Preparation

The lyophilized standard was reconstituted with deionized water to yield the stock standard. The standard was allowed to equilibrate for 15 minutes before making dilutions and vortexed gently to mix. From the stock, the working standard was prepared and the serial dilutions of the standard were prepared with assay diluent at six different concentrations. The standard curve was plotted using the software Softmaxpro (Molecular devices, USA) with TNF- α concentration on the x-axis and absorbance on the y-axis.

Assay Procedure for test samples

1. 96-well microtitre plates were coated with 100 µl per well of diluted capture antibody. Then the plates were sealed and incubated overnight at 4^0C.

2. Wells were aspirated and washed 3 times with wash buffer. After last wash, plates were inverted on absorbent paper to remove the residual buffer.

3. 200 µl/well assay diluent was added and incubated at room temperature for 1 hour.

4. After incubation, wells were aspirated and washed again with wash buffer for three times.

5. The standards and samples were prepared in assay diluent.

6. 100 µl of each standard, sample (24 hour supernatant from whole blood assay), and control were added into appropriate wells. Plates were sealed and incubated overnight at 4^0C.

7. Wells were aspirated and washed again five times with wash buffer.

8. 100 µl of prepared working detector (detection antibody + avidin-HRP reagent) was added to each well and the plates were sealed and incubated for 1 hour at RT.

9. Wells were aspirated and washed again seven times with wash buffer. In this final wash, wells were soaked in wash buffer for 30 seconds to 1 minute for each wash.

10. 100 µl of substrate solution (equal volume of tetramethylbenzidine (TMB) and hydrogen peroxide prepared with assay diluent) was added to each well. Plates were incubated (without plate sealer) for 30 minutes at room temperature in the dark.

11. Finally 50 µl of stop solution was added to each well.

12. The absorbance was read at 450nm within 30 minutes from adding stop solution.

Calculation of Results

The mean absorbance for each set of duplicate standards, controls and samples were calculated. The TNF-α concentrations of the unknown samples were assayed from the standard curve using the software Softmaxpro (Molecular devices, USA). For diluted samples TNF-α concentrations were multiplied by the dilution factor.

Capture antibody preparation

The capture antibody was added at 1:250 dilution to the coating buffer.

Working Detector Preparation

Detection antibody and enzyme reagent were added at 1:250 dilution to assay diluent and vortexed well.

Standards Preparation

The lyophilized standard was reconstituted with deionized water to yield the stock standard. The standard was allowed to equilibrate for 15 minutes before making dilutions and vortexed gently to mix. From the stock, the working standard was prepared and the serial dilutions of the standard were prepared with assay diluent at six different concentrations. The standard curve was plotted using the software Softmaxpro (Molecular devices, USA) with IFN- γ concentration on the x-axis and absorbance on the y-axis.

Assay Procedure for test samples

1. 96-well microtitre plates were coated with 100 µl per well of diluted capture antibody. Then the plates were sealed and incubated overnight at 4^0C.
2. Wells were aspirated and washed 3 times with wash buffer. After last wash, plates were inverted on absorbent paper to remove the residual buffer.

3. 200 µl/well assay diluent was added and incubated at room temperature for 1 hour.

4. Wells were aspirated and washed again with wash buffer for three times.

5. The standards and samples were prepared in assay diluent.

6. 100 µl of each standard, sample (96 hour supernatant from whole blood assay), and control were added into appropriate wells. Plates were sealed and incubated overnight at 4^0C.

7. Wells were aspirated and washed again five times with wash buffer.

8. 100 µl of prepared working detector (Detection antibody + avidin-HRP reagent) was added to each well and the plates were sealed and incubated for 1 hour at RT.

9. Wells were aspirated and washed again seven times with wash buffer. In this final wash, wells were soaked in wash buffer for 30 seconds to 1 minute for each wash.

10. 100 µl of substrate solution (equal volume of tetramethylbenzidine (TMB) and hydrogen peroxide prepared with assay diluent) was added to each well. Plates were incubated (without plate sealer) for 30 minutes at room temperature in the dark.

11. Finally 50 µl of stop solution was added to each well.

12. The absorbance was read at 450nm within 30 minutes from adding stop solution.

Calculation of Results

The mean absorbance for each set of duplicate standards, controls and samples were calculated. The IFN-γ concentrations of the unknown samples were assayed from the standard curve using the software Softmaxpro (Molecular devices, USA). For diluted samples, IFN-γ concentrations were multiplied by the dilution factor.

Intracellular growth kinetics of *Mycobacterium tuberculosis* H37Rv

To determine the intracellular *M. tuberculosis* growth, cultured macrophages were infected with low concentrations of viable bacilli (CFU) to mimic natural infection. Infection ratio was designed in a manner based on that described by Ming Zhang *et al* (158). The multiplicity of infection followed was one CFU to 80 viable macrophages.

After 3 hours, the supernatants were aseptically aspirated and the adhered cells were lysed by treating with distilled water for 10 minutes, serially diluted at 1 in 10 concentration and plated on middlebrook 7H11 agar plates supplemented with OADC (BD, USA). This time point was considered as day-0.

Viable count on day-3 was assessed in the mixture of lysates and supernatants to reveal intracellular multiplication of bacilli and the same procedure was repeated on day-7 also. The seeded plates were sealed and incubated at 37^0C under 5% CO_2. The colony forming units were counted on agar plates after 3 weeks and expressed in log_{10} units.

All the descriptive statistics were expressed as mean ± standard deviation. Differences between the uninfected controls and the infected samples of the same group were analysed by paired Student 't' test. Comparison between the study groups were analysed by Unpaired Student 't' test. The growth kinetics of the bacilli in macrophages between the study groups were analysed by oneway ANOVA and the multiple comparison within the groups were revealed by Tukey Post Hoc tests.

- Results

Measurement of phagocytosis

Phagocytic ability of macrophages was assessed by microscopic observation. The fields were observed to count both bacilli engulfed-macrophages and bacilli free-macrophages. A photo micrographic image of *M. tuberculosis* infected macrophages of a study subject is shown in fig-18. Each time 100 fields were examined for counting and the phagocytic index was expressed in percentage by using the formula described in methods. The mean percentages of phagocytic index of all the four study groups are shown in fig -19. The mean percentages (±SD) were 45.1(±10.3), 27.8(±7.6), 49.8(±8.3) and 24.4(±11.2) for the four study groups vaccinated reactors, vaccinated non-reactors, non-vaccinated reactors and non-vaccinated non-reactors respectively.

When the capacity to uptake/engulf the bacilli by the macrophages were compared between vaccinated and non-vaccinated non-reactors, there was no significant difference.

When compared between vaccinated reactors and vaccinated non-reactors the engulfment was significantly higher among the vaccinated reactors ($P < 0.05$).

When compared between vaccinated and non-vaccinated reactors, there was no significant difference.

When compared between vaccinated non-reactors and non-vaccinated reactors, the engulfment was significantly higher among the non-vaccinated reactors ($P < 0.05$).

Superoxide dismutase (SOD) assay

The SOD activity was determined by the equation and expressed as SOD-525 units as described in methods. The mean SOD-525 units secreted in all the four study groups were estimated along with their controls. The results of the SOD secretion for the four study groups are shown in fig -20.

The mean (±SD) secretion of SOD-525 units in the supernatant of uninfected control wells were 9.58 ± 2.72, 8.47 ± 2.54, 8.83 ± 6.34 and 7.28 ± 3.04 for the four study groups vaccinated reactors, vaccinated non-reactors, non-vaccinated reactors and non-vaccinated non-reactors respectively. In the infected wells the SOD levels were 13.80 ± 3.23, 12.97 ± 6.59, 13.64 ± 7.02 and 10.67 ± 3.53 units for the four study groups respectively. In all the four groups, the supernatant with infection showed a significant increase when compared to their respective uninfected control supernatant ($p < 0.05$).

When the levels of SOD were compared between vaccinated and non-vaccinated non-reactors, there was no significant difference.

When compared between vaccinated reactors and vaccinated non-reactors there was no significant difference.

When compared between vaccinated and non-vaccinated reactors, there was no significant difference.

When compared between vaccinated non-reactors and non-vaccinated reactors, there was no significant difference.

Glutathione (GSH) assay

Assay for standard

The standard curve for glutathione was derived using spectrophotometry as described in methods. The five different concentrations that cover the range of 20-100 μmol/L of glutathione and their corresponding O.D. values are shown in fig-21. The glutathione levels of the test samples of the study subjects were derived from this standard curve.

Assay for test samples

Intracellular glutathione levels of the test samples (macrophage lysates) for the four respective groups along with their controls were assayed spectrophotometrically and derived from the standard graph as described in methods. The results of the glutathione secretion for the four study groups are shown in fig–22.

The mean (±SD) glutathione level (nmol/mg of protein) in uninfected control macrophage lysates were 3.455 ± 0.581, 2.91 ± 0.598, 2.973 ± 0.552 and 2.431 ± 0.225 for the four groups vaccinated reactors, vaccinated non-reactors, non-vaccinated reactors and non-vaccinated non-reactors respectively. In infected macrophage cultures the glutathione levels were 4.065 ± 0.499, 3.448 ± 0.540, 4.144 ± 0.688 and 2.997 ± 0.404 nmol/mg of protein for the four groups respectively. In all the four study groups, infection with *M. tuberculosis* H37Rv had resulted in a significant increase in intracellular glutathione when compared to their respective uninfected control samples ($p<0.05$).

When the levels of glutathione were compared between vaccinated and non-vaccinated non-reactors, the glutathione level was significantly higher among vaccinated non-reactors ($P < 0.05$).

When compared between vaccinated reactors and vaccinated non-reactors, the glutathione level was significantly higher among the vaccinated reactors ($P < 0.05$).

When compared between vaccinated and non-vaccinated reactors, there was no significant difference.

When compared between vaccinated non-reactors and non-vaccinated reactors, the level was significantly higher among non-vaccinated reactors ($P < 0.05$).

Estimation of TNF-α

The TNF-α level in the whole blood supernatant was estimated at 24 hour post infection by ELISA for all the four study groups along with their controls as described in methods. The average results of the TNF- α level for the study groups are shown in fig -23.

The mean (±SD) TNF- α level (pg/ml) in uninfected culture supernatants were 698 ± 35.8, 582.73 ± 50.01, 725.56 ± 36.7 and 541.11 ± 68.1 for the four groups vaccinated reactors, vaccinated non-reactors, non-vaccinated reactors and non-vaccinated non-reactors respectively. In infected whole blood the TNF- α levels were 2294 ± 421.22, 1723.64 ± 179.18, 2495.56 ± 545.11 and 1574.44 ± 235.62 pg/ml for the four groups respectively. In all the four study groups, the infected whole blood showed a significant increase when compared to their respective uninfected samples ($p<0.05$).

When the levels of TNF-α were compared between vaccinated and non-vaccinated non-reactors, there was no significant difference.

When compared between vaccinated reactors and vaccinated non-reactors the level was significantly higher among the vaccinated reactors ($P < 0.05$).

When compared between vaccinated and non-vaccinated reactors, there was no significant difference.

When compared between vaccinated non-reactors and non-vaccinated reactors, the level was significantly higher among the non-vaccinated reactors ($P < 0.05$).

Estimation of IFN-γ

The IFN-γ level in the whole blood supernatant was estimated at 96 hour post infection by ELISA for all the four study groups along with their controls as described in methods. The average results of the IFN- γ level for the study groups are shown in fig -24.

The mean (±SD) IFN- γ responses (pg/ml) in uninfected culture supernatants were 592 ± 131.81, 387.27 ± 47.56, 595.56 ± 106.08 and 355 ± 68.28 for the four groups vaccinated reactors, vaccinated non-reactors, non-vaccinated reactors and non-vaccinated non-reactors respectively. In infected whole blood the levels of IFN-γ were 9493 ± 2660.03, 4620 ± 1007.61, 12298.89 ± 6287.51 and 4027.78 ± 1448.04 pg/ml for the four groups respectively. In all the four groups, the infected whole blood showed a significant increase when compared to their respective uninfected samples ($p<0.05$).

When the levels of IFN-γ were compared between vaccinated and non-vaccinated non-reactors, there was no significant difference.

When compared between vaccinated reactors and vaccinated non-reactors the level was significantly higher among the vaccinated reactors ($P < 0.05$).

When compared between vaccinated and non-vaccinated reactors, there was no significant difference.

When compared between vaccinated non-reactors and non-vaccinated reactors, the level were significantly higher among the non-vaccinated reactors ($P < 0.05$).

Intracellular growth kinetics of *Mycobacterium tuberculosis* H37Rv

The growth kinetic pattern of the bacilli in the macrophage host cells of all the four study groups was assessed on day-0, day-3 and day-7 and expressed as log_{10} units. The growth kinetic pattern of the bacilli is shown in fig-25.

The mean log_{10} CFU (±SD) in the cell lysates at day-0 were 2.77 ± 0.05, 2.64 ± 0.11, 2.67 ± 0.14 and 2.52 ± 0.16 for the four study groups vaccinated reactors, vaccinated non-reactors, non-vaccinated reactors and non-vaccinated non-reactors respectively. The mean log_{10}CFU (±SD) at day-3 for the four study groups were 3.39 ± 0.15, 3.53 ± .06, 3.50 ± 0.11 and 3.41 ± 0.11 respectively. The mean log_{10}CFU (±SD) at day-7 for the four study groups were 3.62 ± 0.14, 3.79 ± 0.14, 3.73 ± 0.11 and 3.75 ± 0.16 respectively.

When the growth kinetic patterns of the bacilli were compared between vaccinated and non-vaccinated non-reactors, growth was significantly lower among the non-vaccinated non-reactors on day-0 and day-3 ($P < 0.05$). But on day-7, there was no significant difference observed between the groups.

When compared between vaccinated reactors and vaccinated non-reactors, growth was significantly lower among the vaccinated reactors on day-3 and on day-7 ($P < 0.05$).

When compared between vaccinated and non-vaccinated reactors, there was no significant difference observed on all the three days.

When compared between vaccinated non-reactors and non-vaccinated reactors, there was no significant difference on all three days.

PHAGOCYTOSIS OF *M. TUBERCULOSIS*

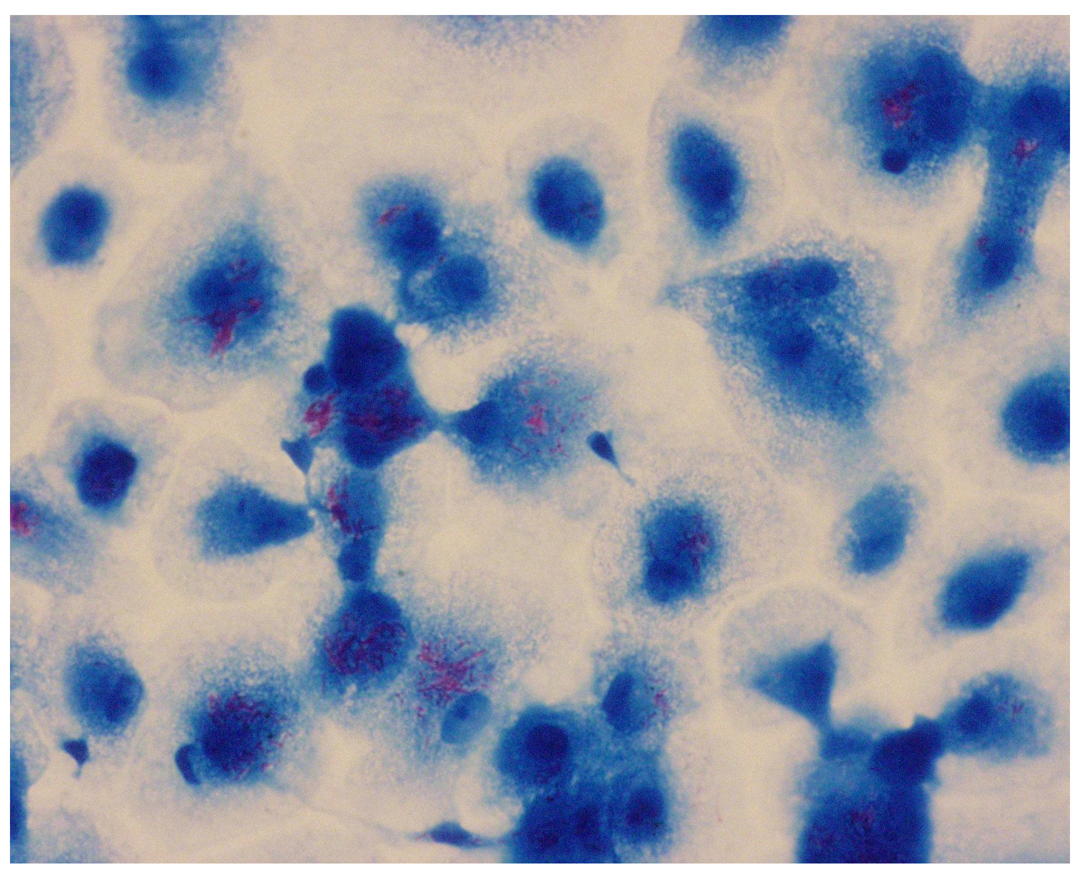
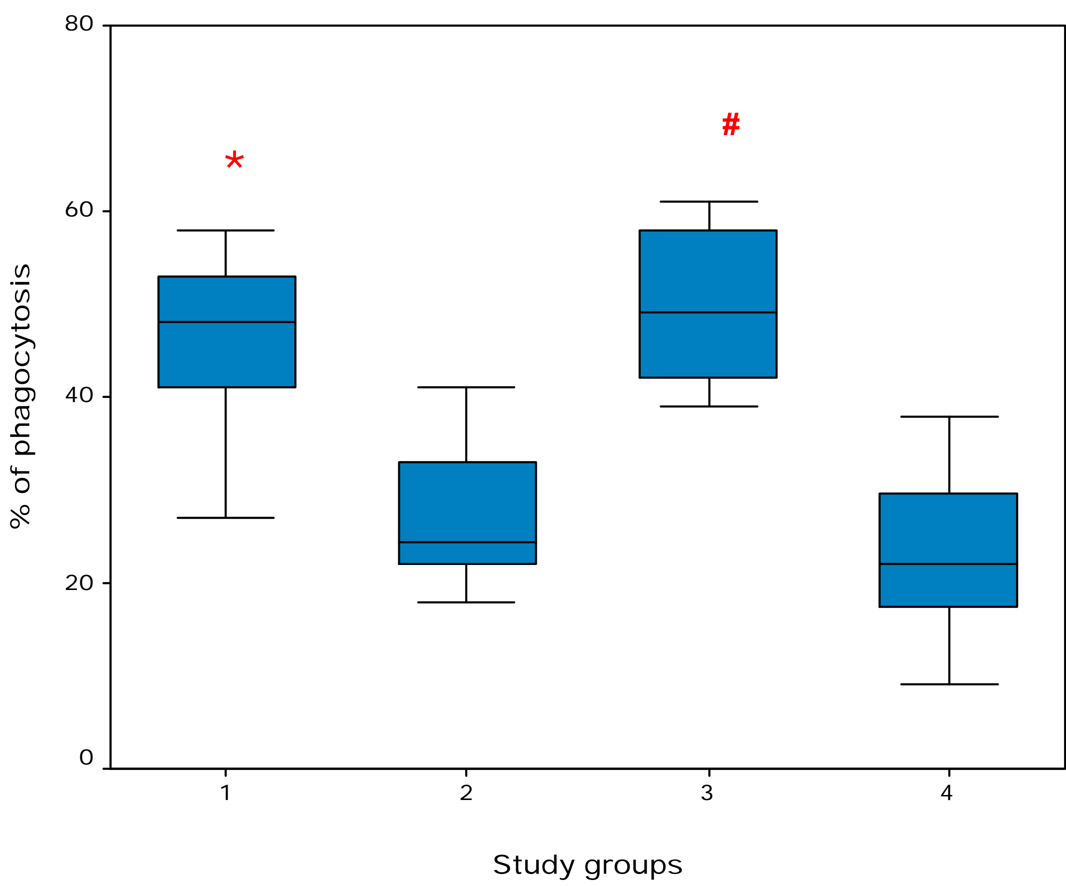


Fig - 18. **Photomicrograph showing *M. tuberculosis* infected macrophages (X100).** Macrophages layered on coverslips were infected with *M. tuberculosis* H37Rv at multiplicity of infection of 10:1 and stained with Kinyoun's acid fast staining method to reveal the bacilli-engulfed macrophages and bacilli-free macrophages.

MEASUREMENT OF PHAGOCYTOSIS



1 – Vaccinated reactors (n=9) **2 – Vaccinated non-reactors (n=14)**

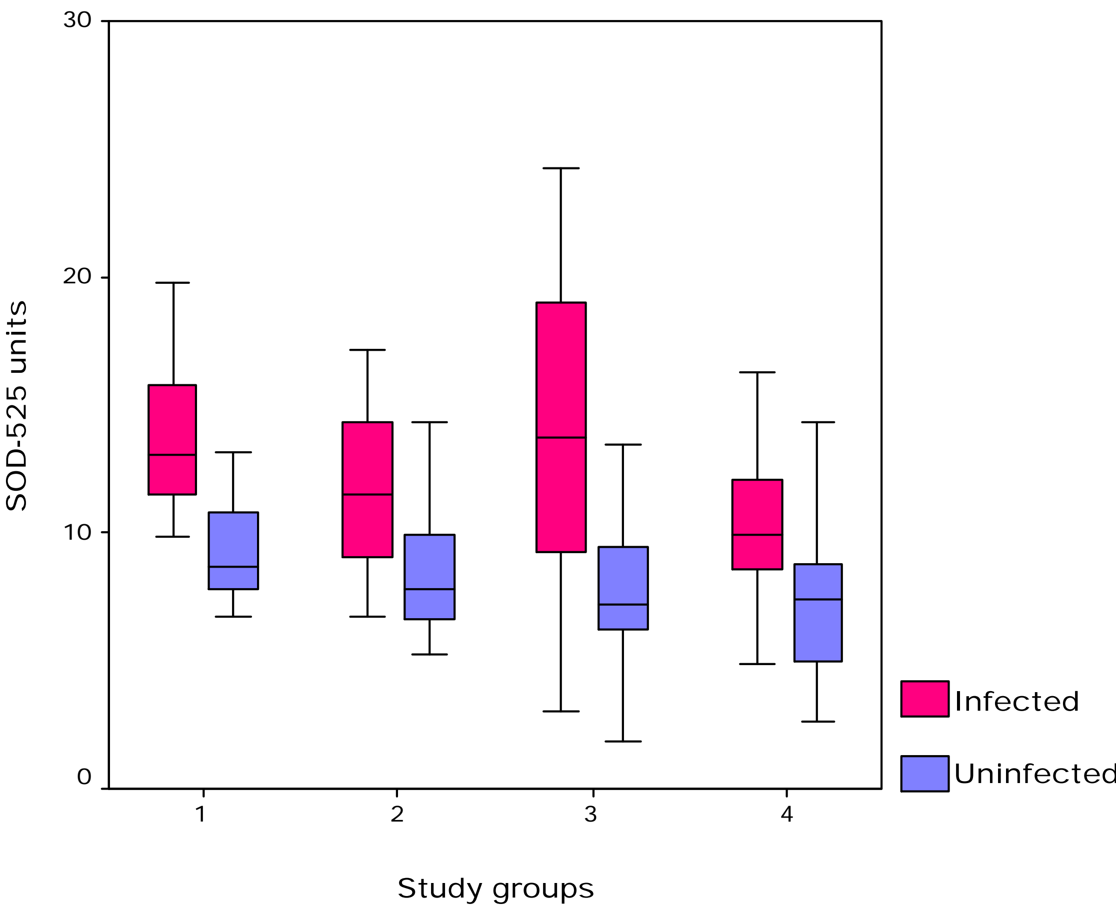
3 – Non-vaccinated reactors (n=9) **4 – Non-vaccinated non-reactors (n=15)**

Fig - 19. Measurement of phagocytosis in the study groups. Phagocytic ability of macrophages was assessed by microscopic observation. Each time 100 fields were examined for counting and the phagocytic index was expressed in percentage.

* When compared between vaccinated reactors and vaccinated non-reactors the engulfment was significantly higher among the vaccinated reactors **($P < 0.05$)**.

\# When compared between vaccinated non-reactors and non-vaccinated reactors, the engulfment was significantly higher among the unvaccinated reactors **($P < 0.05$)**.

SUPEROXIDE DISMUTASE (SOD) ASSAY



1 – Vaccinated reactors (n=11) **2 – Vaccinated non-reactors (n=18)**

3 – Non-vaccinated reactors (n=10) **4 – Non-vaccinated non-reactors (n=18)**

Fig - 20. Superoxide dismutase (SOD) assay in the study groups. In all the four groups, the supernatant of infected wells yielded significantly higher levels of SOD when compared to their respective uninfected samples. But the comparison between study groups did not show any significant difference.

GLUTATHIONE (GSH) STANDARD CURVE

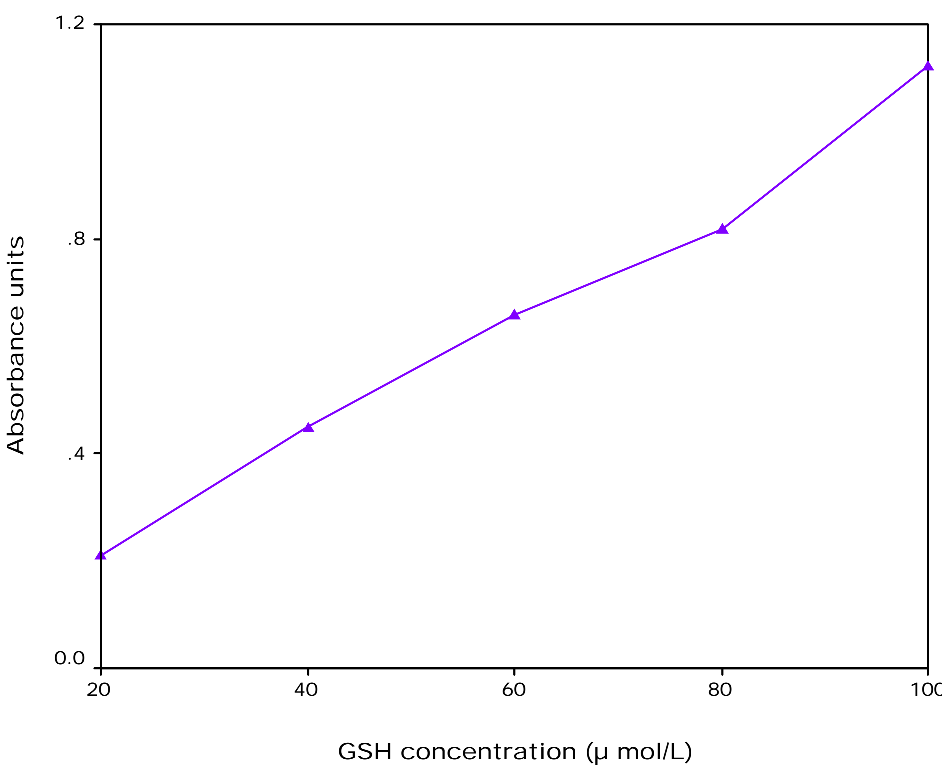
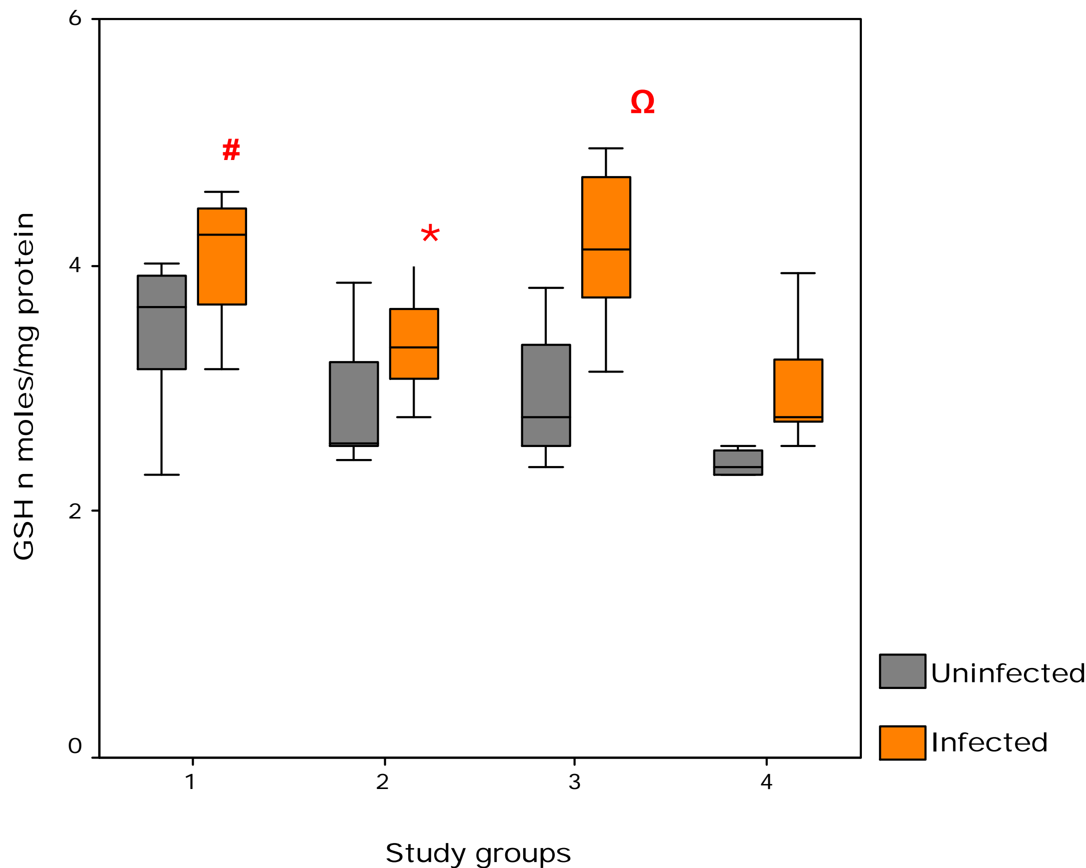


Fig - 21. **Glutathione standard curve at 400nm.** The concentrations of glutathione were prepared by using 5% meta-phosphoric acid. The spectrophotometric readings were the averages of results performed in triplicate.



1 – Vaccinated reactors (n=10) **2 – Vaccinated non-reactors (n=15)**

3 – Non-vaccinated reactors (n=9) **4 – Non-vaccinated non-reactors (n=14)**

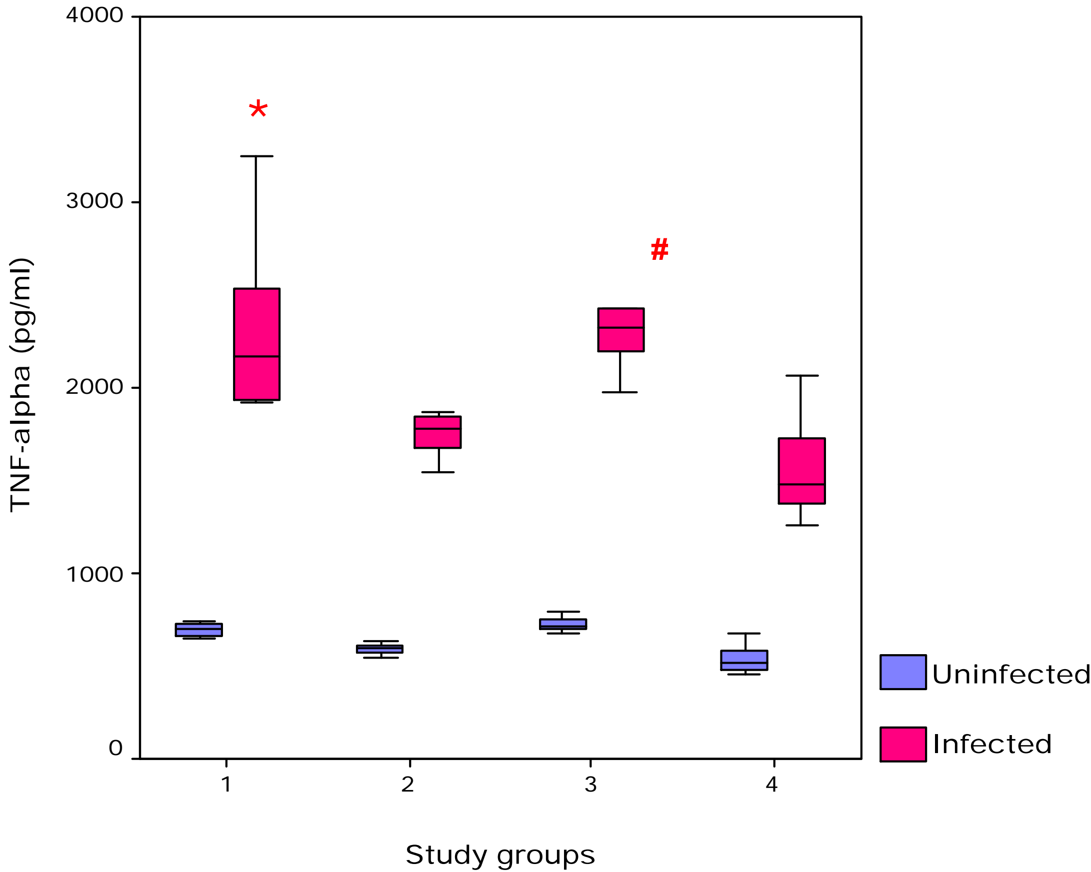
Fig - 22. **Glutathione assay in the study groups.** In all the four study groups, infection with *M. tuberculosis* H37Rv yielded significantly higher glutathione levels when compared to their respective uninfected control samples.

* When the levels of glutathione were compared between vaccinated and unvaccinated non-reactors, the glutathione level was significantly higher among vaccinated non-reactors **($P < 0.05$)**.

When compared between vaccinated reactors and vaccinated non-reactors, the glutathione level was significantly higher among the vaccinated reactors **($P < 0.05$)**.

Ω When compared between vaccinated non-reactors and non-vaccinated reactors, the level was significantly higher among non-vaccinated reactors **($P < 0.05$)**.

ESTIMATION OF TNF-α



1 – Vaccinated reactors (n=10) **2 – Vaccinated non-reactors (n=11)**

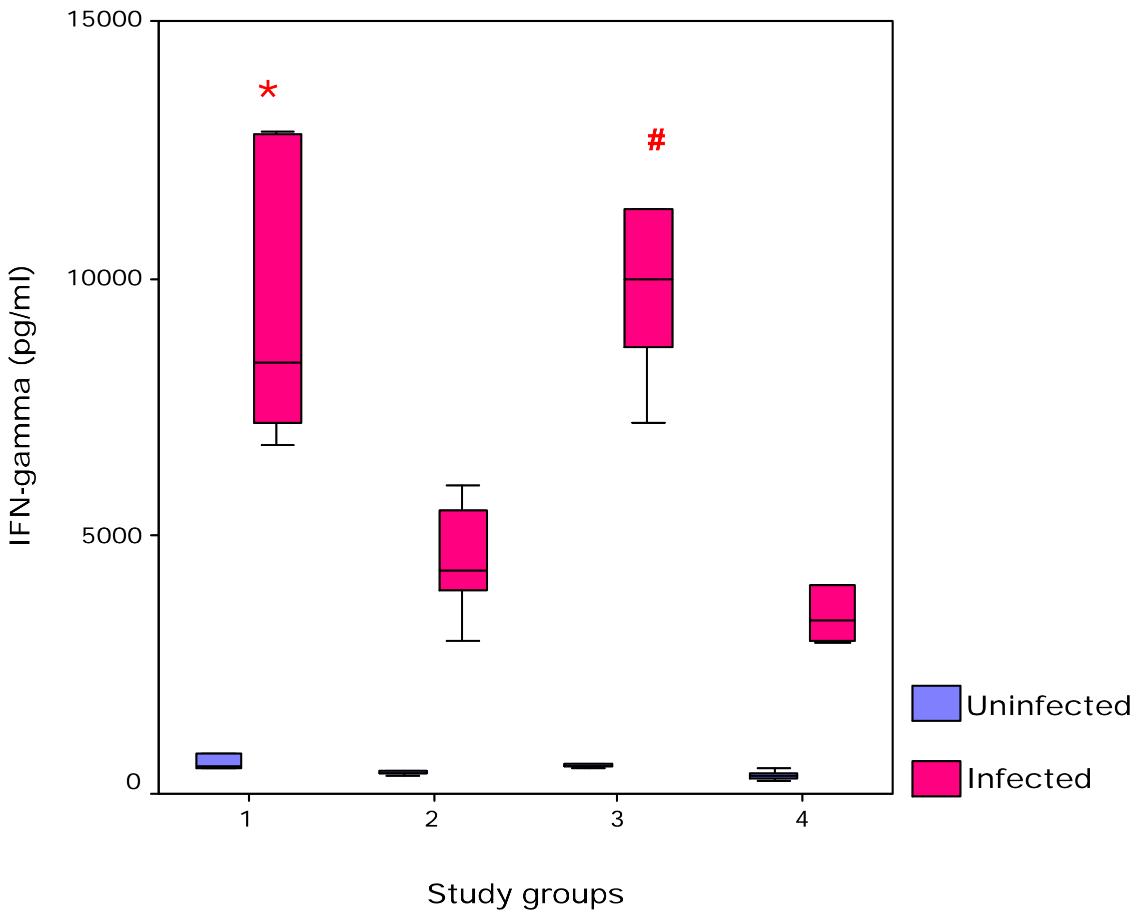
3 – Non-vaccinated reactors (n=9) **4 – Non-vaccinated non-reactors (n=18)**

Fig - 23. **Estimation of TNF- α from whole blood cultures at 24 hour post-infection in the study groups.** In all the four groups the infected whole blood yielded significantly higher levels of TNF- α alpha when compared to their respective uninfected samples.

* When compared between vaccinated reactors and vaccinated non-reactors the level was significantly higher among the vaccinated reactors **($P < 0.05$)**.

\# When compared between vaccinated non-reactors and non-vaccinated reactors, the level was significantly higher among the non-vaccinated reactors **($P < 0.05$)**.

ESTIMATION OF IFN-γ



1 – Vaccinated reactors (n=10) **2 – Vaccinated non-reactors (n=11)**

3 – Non-vaccinated reactors (n=9) **4 – Non-vaccinated non-reactors (n=18)**

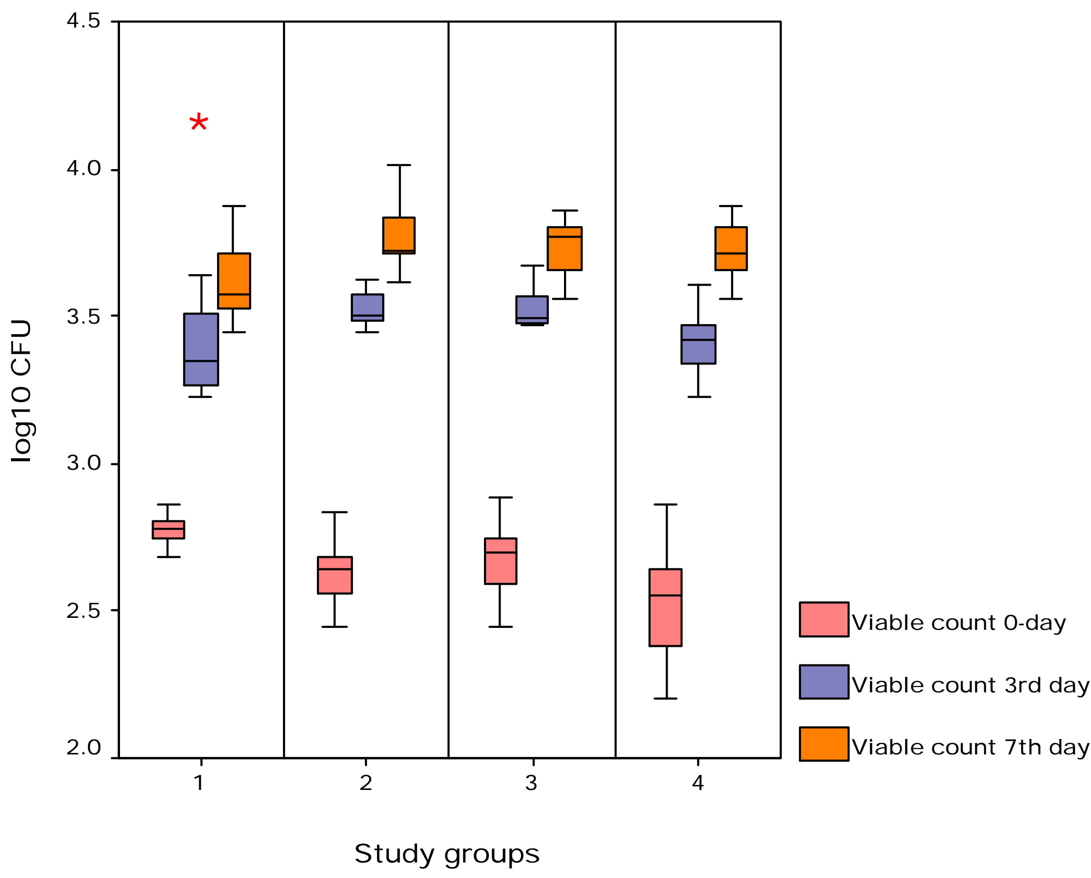
Fig - 24. **Estimation of IFN-γ from whole blood cultures at 96 hour post-infection in the study groups.** In all the four groups the infected whole blood yielded significantly higher levels of IFN-γ when compared to their respective uninfected samples.

* When compared between vaccinated reactors and vaccinated non-reactors the level was significantly higher among the vaccinated reactors **($P < 0.05$)**.

\# When compared between vaccinated non-reactors and non-vaccinated reactors, the level was significantly higher among the non-vaccinated reactors **($P < 0.05$)**.

INTRACELLULAR GROWTH KINETICS OF

MYCOBACTERIUM TUBERCULOSIS H37RV



1 – Vaccinated reactors (n=9) **2 – Vaccinated non-reactors (n=13)**

3 – Non-vaccinated reactors (n=8) **4 – Non-vaccinated non-reactors (n=13)**

Fig - 25. **Intracellular growth kinetic pattern of *Mycobacterium tuberculosis* H37Rv in the study groups.** The growth kinetic patterns of the bacilli were expressed in $\log_{10}$ units. The values shown are the mean values of the four study groups on the respective days.

*

***The growth kinetic pattern of* M. tuberculosis *H37Rv on the respective days showed a significant difference among the vaccinated reactors when compared to the vaccinated non-reactors** ($P < 0.05$).*

There was no significant difference between other groups.

Measurement of Phagocytosis

- The capacity to uptake/engulf the bacilli by the macrophages was not significantly higher among the vaccinated subjects. The engulfment was significantly higher among reactors irrespective of BCG vaccination.

Superoxide dismutase (SOD) assay

- The secretion of SOD was not significantly higher among the vaccinated subjects. Similarly tuberculin response also did not influence the SOD secretion.

Glutathione (GSH) assay

- The secretion of glutathione by macrophages was significantly higher among the vaccinated non-reactors when compared to the non-vaccinated non-reactors. But among reactors there was no significant difference observed between vaccinated and non-vaccinated subjects. The secretion was significantly higher among the reactors irrespective of BCG vaccination.

Estimation of TNF-α

- The secretion of TNF-α was not significantly higher among vaccinated subjects. The secretion was significantly higher among reactors irrespective of BCG vaccination.

Estimation of IFN-γ

- The secretion of IFN-γ was not significantly higher among vaccinated subjects. The secretion was significantly higher among reactors irrespective of BCG vaccination.

Intracellular growth kinetics

- The growth kinetics patterns of M. tuberculosis H37Rv on the respective days showed a significant difference among the vaccinated reactors when compared to the vaccinated non-reactors. There was no significant difference between other groups.

- Discussion

In India, as elsewhere in the world, BCG vaccination is given during neonatal period to provide protection against tuberculosis, particularly the more severe forms of childhood tuberculosis such as miliary and meningeal tuberculosis. But the exact immune response elicited after this neonatal BCG vaccination within the human host is still not clear and has been the subject of research for the past three decades.

Information regarding the effect of neonatal BCG vaccination and the influence of tuberculin reactivity at intracellular level in adolescent and young adult period is very important, since TB affects a large number of young people (152). These kinds of studies may provide important insights into the immunogenic activity of the BCG vaccine. Besides, such studies can help to identify a marker for adequate immunity against tuberculosis, as in the present state of knowledge such a marker is not available.

In this immuno-epidemiological study, subjects were drawn from three different study areas; Urban, sub-urban and rural in and around Chennai city (South India) to study the descriptive epidemiological pattern of neonatal BCG vaccination, tuberculin response and the common risk factors associated with the tuberculin skin test reactivity. Later, immunological macrophage culture experiments were performed to assess the influence of neonatal BCG vaccination and tuberculin response on anti-mycobacterial activity of macrophages.

Three different kinds of areas (urban, suburban and rural) were selected as this will represent the whole population. All the three areas were selected for the following reasons – the distance (not far away) from the cell culture laboratory, transportation convenience, transportation time duration and

cooperation of the volunteers. Moreover no major TB related surveys or studies have been carried out in urban Chennai and sub-urban Pallavaram. Rural Tirupandiyur was purposefully taken, as it is a part of the major South Indian BCG trial area.

In several previous field studies, BCG scar status was routinely used as a surrogate marker of vaccination or of effective vaccination (159, 160, 161, 162). In the present study population, 43% of the subjects were BCG vaccinated as assessed by the presence of BCG scar. The area-wise BCG-scar prevalence was 52%, 44% and 14% for urban, sub-urban and rural study areas respectively. Majority of the vaccinated study subjects (88%) had the scar size of 1-10mm and a few subjects (12%) had the size of above 10mm. It is interesting to note that all the BCG scars in the rural BCG trial area (Tirupandiyur) were less than 10mm. The distribution of scar sizes was not significantly different according to gender in all the three study areas.

In this study, two doses of PPDs (1 TU and 10 TU) were administrated simultaneously on both of the forearms of the volunteers to assess the impact of the dose of PPD on tuberculin skin test. This study showed that there was no significant difference between the indurations of PPD-1 TU and PPD-10 TU. The higher dose of PPD did not reveal higher detection rate when compared to the lower dose used. So in this study the indurations of PPD-1 TU alone were used in the interpretation of the results. Moreover this PPD-1 TU dose was recommended in India for standard tuberculin skin test (Mantoux test) as per earlier recommendation of WHO (157). A study carried out by Chadha *et al* in India has also suggested that usage of PPD 1 TU rather than higher strengths was better in separating the infected individuals from the community (163).

Moreover, in general all those reactive for PPD-1 TU must be reactive to PPD-10 TU. But the result here is different. Unexpectedly some of the study subjects (13%) were reactive to PPD-1 TU but non-reactive on PPD-10 TU. This same unexpected result was also obtained in a study conducted in Australia to reveal a paired comparison of tuberculin skin test measurements using 5 TU and 10 TU. Some PPD-5 TU reactors of the study did not react to PPD-10 TU (164). As mentioned in results, the reasons for this anomaly cannot be explained at this time, and needs to be studied further.

The influence of neonatal BCG vaccination on the tuberculin response was analysed in all the three study areas. The induration size of 12mm was used as the cut-off point in this study to classify the study subjects into reactors and non-reactors. This same cut-off point was obtained/used in a study conducted to compare two different skin test antigens RT-23 and PPD-S in the study area of Chingleput BCG trial (165). This ≥12mm anti-mode was also used in the previous Chingleput major BCG trial and other TB incidence surveys by ICMR (111, 166). One of the present study areas, Tirupandiyur is a part of Chingleput major BCG trial and other two areas are also adjacent to the trial area.

In the present study the prevalence of reactors was 31 percent. The proportion of tuberculin response with BCG scar status has revealed a statistical significance ($P < 0.05$) in the sub-urban and rural ($P = 0.05$) subjects but there was no significance among the urban study subjects. If we look at it gender wise, the significance was found only among males and not among females.

Previous studies carried out to analyze the relationship between BCG vaccination and tuberculin response have showed variable results; some showed a relationship and some studies did not. The effect of BCG vaccination on tuberculin response was analyzed in eleven different surveys by Menzies D *et al* and revealed no relationship between the tuberculin response after BCG vaccination and the protective efficacy of the vaccine (167). Other studies conducted at different parts of the world have also showed no relationship between BCG vaccination and tuberculin response (168, 169, 170).

But a meta-analysis revealed that the individuals who had received BCG vaccination were more likely to have a positive skin test (171). Another study that supports the influence of neonatal BCG vaccination on the tuberculin response was conducted by Centre for prevention and control of tuberculosis in Spain and revealed that BCG vaccination at birth and for school age children caused reactivity to tuberculin even after 20 to 25 years (172).

Snider *et al* have proposed that the effect of prior BCG vaccination on the tuberculin skin test varies depending on age at vaccination, interval between vaccination and tuberculin skin testing, the strain of BCG vaccine used, skin testing reagents used and geographical location of the population being tested (173).

In a study carried out by Tuberculosis Research Centre (Unpublished data), it was seen that the proportion of reactors following BCG was high only up to 18 months after vaccination and thereafter it was not different from the proportion of reactors in the unvaccinated.

Apart from neonatal BCG vaccination, the tuberculin response depends upon numerous other factors. This study has evaluated the association of those other risk factors also with the tuberculin response. The risk factor of 'Family member (or close contact) with TB' was significantly associated (P=0.05) with the tuberculin reactivity. All the other risk factors such as prolonged fever, persistent cough etc were not significantly associated with the tuberculin reactivity in the present study. A previous study that assessed the association of risk factors with tuberculin reactivity through a 'risk assessment questionnaire' was carried out by Koppaka *et al* and have showed that 'Close contact with a TB patient' and 'Birth in a high risk country' were associated with the tuberculin reactivity (174).

In the immunological experiment part, macrophage killing profile was assessed for the volunteers from the four natural study groups. There is very limited information on the immunological aspect of the BCG vaccine at intracellular level in terms of prevention of primary infection with *M. tuberculosis* and in the prevention of the development of disease. It is important to identify the cellular level differences in the susceptibility of the community to tuberculosis particularly with reference to BCG.

Anti-mycobacterial immune defenses are primarily mediated by macrophages and T-lymphocytes. Alveolar macrophages are the primary cell type involved in the initial uptake of *M. tuberculosis* and this phagocytosis process induces the host immune system to produce a characteristic pattern of cytokines that have potent immuno-regulatory effects (28). Thus, macrophages play a key role in the initiation and direction of cell-mediated immune response. Modulation of immunity after BCG vaccination is considered as the vectorial sum of its many effects on

influx, trapping and activation of macrophages, lymphocytes and other immune cells (145).

Several studies were performed to assess the effect of BCG vaccination and its influence on the immune cells such as macrophages, T cells etc (144, 145, 175, 176, 177, 178, 179, 180). In this study, the influence of neonatal BCG vaccination and the effect of tuberculin reactivity on the intracellular killing mechanism of macrophages were assessed by various parameters of the killing profile such as; macrophage capacity to uptake/engulf the bacilli, SOD production during early interaction, glutathione secretion by macrophages, TNF-α and IFN-γ secretion by the host and the ability of macrophage to control the intracellular multiplication of *M. tuberculosis*.

Phagocytic index was measured in this study because the infectious process of *M. tuberculosis* initially involves the adherence of the bacilli to the surface of macrophage cells. These macrophages play a dual role in tuberculosis, promoting not only the protection against the disease but also the survival of the pathogen. In the present study, the capacity to uptake/engulf the bacilli by the macrophages was not enhanced by BCG vaccination. But the engulfment was significantly higher among reactors irrespective of BCG vaccination. A technical problem in this experiment was using acid fast staining method we could not distinguish the bacilli which were phagocytosed or adhered. But the 'adherence/binding' was also considered because it is also a step (probably, the first) involved in the phagocytic process.

Macrophages produce reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) that have potent antimicrobial activity. The effective role of ROI and RNI were previously studied in mouse macrophages (54, 181). Despite the toxic effects of ROI and RNI *M. tuberculosis* can survive and grow within human macrophages. This resistance to killing by macrophages is critical to the virulence of *M. tuberculosis* to establish an infection and disease. The production of ROI is initiated by NADPH oxidase, which catalyzes the reduction of molecular oxygen to superoxide (O_2^-) (182). This superoxide is highly toxic to *M. tuberculosis*. To overcome this killing mechanism, the bacilli produce superoxide dismutase (SOD) proteins which convert the toxic superoxide into molecular oxygen and hydrogen peroxide and thereby contribute to the survival of the bacilli (183). The contribution of superoxide dismutase in the survival of *M. tuberculosis* was previously carried out in mouse macrophages by Piddington *et al* by using mutant strains of *M. tuberculosis* that are defect in SOD production. These strains were readily killed by ROI and proved that superoxide dismutase contributes to the resistance of *M. tuberculosis* against oxidative burst products generated by activated macrophages (184). In this study, SOD secretion during infection was measured to determine the ability of *M. tuberculosis* to survive within the host cells. The secretion of SOD level was not significantly altered by neonatal BCG vaccination and the tuberculin reactors also did not show any significant difference when compared to non-reactors.

In this study, the level of glutathione against tuberculosis infection was assessed by the estimation of intracellular glutathione levels in the macrophage lysates. Glutathione is a tripeptide composed of glutamate,

cysteine and glycine. It is an anti-oxidant that plays a vital role in cellular detoxification and enhancement of immune functions. This glutathione is believed to have an important role in RNI mediated killing by the macrophages. A hypothesis proposed by Vishwanath *et al* states that, nitric oxide (NO) reacts with glutathione (GSH) to form S-nitrosoglutathione (GSNO) and this formation increases the activity of NO. The growth of intracellular mycobacteria, including *M. tuberculosis* may be controlled by glutathione and/or S-nitrosoglutathione (GSNO) generated by macrophages (185). Previous experiments have showed that mycobacteria are sensitive to glutathione at the concentration of above 5mM (186) and a mutant BCG strain which is defective in glutathione transport was partially resistant to the toxic effects of glutathione (187). An experiment to reveal the role of glutathione in macrophage control of mycobacteria in J774 cells and human macrophages has showed treatment of human macrophages with N-acetyl cysteine resulted in significant increase in glutathione level and significant killing of BCG strains (185). Hence it becomes important to measure the levels of glutathione secreted by macrophages during infection. The level of glutathione was assayed in all four study groups. The secretion of glutathione by macrophages was significantly higher among the vaccinated non-reactors when compared to the unvaccinated non-reactors. But when compared with the unvaccinated reactors the secretion was significantly low. So apart from BCG vaccination, tuberculin reactivity has also influenced the glutathione secretion.

In this study, whole blood assay was used as a tool to measure the secretion of cytokines TNF-α and IFN-γ. This whole blood culture model was found to be a useful tool in studying the immunogenecity of the BCG vaccination

(188) and to evaluate the aspects of immune functions such as killing of bacteria and expression of cytokines (189, 190, 191). The role of TNF-α and IFN-γ in the protection of tuberculosis was reviewed by several studies (59, 60). TNF-α is synthesized in and released from macrophages in response to *M. tuberculosis* infection within the macrophage. IFN-γ is produced by T cells and has many functions in the macrophage activation. In the present study, the secretion of TNF-α and IFN-γ were not significantly enhanced by BCG vaccination. But the secretions were significantly higher among reactors irrespective of BCG vaccination. A drawback in whole blood culture model is, in whole blood, different cells secrete cytokines apart from macrophages and T-cells. But practically it was very difficult to enumerate the total count of all cells and to identify the exact source-cells for the particular cytokine secretion. In this study, the secreted cytokine level was considered as the ‘overall immune marker’ of the subjects.

The ability of *M. tuberculosis* to multiply within the macrophage host cell is an important factor in the pathology of tuberculosis. The mechanism underlying the differential growth rates of *M. tuberculosis* in macrophages remain speculative. Many attempts were made to define the intracellular burden of the bacilli within mouse and human macrophages (181, 190, 192, 193, 194).

In this study, the *in vitro* growth pattern of *M. tuberculosis* H37Rv within macrophage host cells from the study groups were analysed on 0-day, 3rd day and 7th day. The multiplicity of infection used in this experiment was 1 cfu : 80 macrophage to mimic the natural *in vivo* infection conditions as previously followed by Ming Zhang *et al* (158). The results of the present

experiment have revealed no significant difference in the intracellular growth pattern between the study groups. So, BCG vaccination did not restrict the bacillary growth within the macrophage host cells.

This is the first study that compared the levels of phagocytic index, SOD, glutathione, TNF-α, IFN-γ and the growth kinetics pattern of *M. tuberculosis* H37Rv in the four natural study groups.

The immune response elicited by neonatal BCG vaccination in healthy young adults, by comparing vaccinated and unvaccinated non-reactors did not reveal a significant difference in macrophage killing. Among the vaccinated subjects the immune response of reactors are significantly higher than non-reactors. There is no significant difference among the reactors of both vaccinated and non-vaccinated. So, present study suggests that neonatal BCG vaccination did not enhance the killing capacity of macrophages in adolescent period. Moreover, the immune response of unvaccinated reactors was significantly higher when compared to the vaccinated non-reactors. This indicates that in someway BCG vaccination suppresses the immune response.

Several reviews have addressed the need for newer TB vaccines (195, 196, 197, 198, 199, 200, 201, 202, 203, 204). Different vaccine development strategies were started in the 90s and were genetically modified-BCG vaccines, attenuated strains of *M. tuberculosis*, Non-mycobacterial live vaccines, attenuated mycobacterial species, subunit and DNA vaccines (153).

To develop better vaccines, detailed studies characterizing the protective immune response induced by existing-BCG are important. If BCG vaccination given at birth can give only short-lived immunity, the options are either to replace BCG with a vaccine that gives a longer duration of protection or to design a vaccine that can be given at a later time point to boost existing immunity and provide protection in adults. Both of these approaches have advantages and disadvantages, and the vaccines now entering into clinical trials include proponents of both approaches (200).

The recent advances in the development of new vaccines against tuberculosis have entered into early clinical trials. A recombinant modified vaccinia virus Ankara expressing a major secreted antigen from *M. tuberculosis*, antigen 85A, was the first new tuberculosis vaccine (MVA85A) to enter into clinical trials in September 2002 (205). In a series of phase I clinical trials in the UK and Africa, MVA85A had an excellent safety profile and was highly immunogenic (206, 207, 208). Boosting vaccinations with MVA85A could offer a practical and efficient strategy for enhancing and prolonging anti-mycobacterial immunity in tuberculosis-endemic areas (209, 210).

The potential benefit of existing-BCG and newer TB vaccines need to be reviewed in the light of the epidemiology of tuberculosis. An active programme of research like this on the existing BCG vaccine and its influence on immune response will enhance our understanding of the BCG's mode of action and in addition these kinds of researches will provide an essential background for the development and evaluation of new TB vaccines.

- Summary & Conclusion

An Immuno-epidemiological study was performed to study the effect of neonatal BCG vaccination and tuberculin response on macrophage killing profile.

In epidemiological field work part, the study subjects were drawn from three different study areas; urban, sub-urban and rural in and around Chennai city. The descriptive epidemiological pattern of neonatal BCG vaccination, its impact on tuberculin skin test and certain common risk factors associated with the tuberculin skin test reactivity were studied. Finally the study subjects for the immunological laboratory experiments were recruited and were grouped in to four natural study groups as;

Study Group 1	Vaccinated reactors
Study Group 2	Vaccinated non-reactors
Study Group 3	Non-vaccinated reactors
Study Group 4	Non-vaccinated non-reactors

In Immunological laboratory work part, the elucidation of macrophage killing profile was studied for all the four groups. The parameters used for the macrophage killing profile were; (i) Measurement of phagocytosis, (ii) Superoxide dismutase assay, (iii) Glutathione assay, (iv) Tumour necrosis factor-α assay, (v) Interferon-γ assay and (vi) Intracellular growth kinetics of *Mycobacterium tuberculosis* H37Rv.

This study is the first to investigate and to compare the influence of neonatal BCG vaccination and the effect of tuberculin responses on macrophage killing mechanisms of adolescents from the four natural study groups.

Concluding Remarks

- The proportion of tuberculin response with BCG vaccination has revealed a statistical significance in Sub-urban Pallavaram and rural Tirupandiyur. No significance found in urban Chennai.

- The distributions of tuberculin reaction sizes were almost similar between vaccinated and non-vaccinated subjects.

- The detection level of PPD-10 TU was not superior when compared to PPD-1 TU.

- This risk factor of 'Family member (or close contact) with TB' was significantly associated (*P*=0.05) with the tuberculin reactivity.

- BCG vaccination did not appear to result in a significant increase of the so called protective responses such as phagocytic index, SOD, Glutathione, TNF-α and IFN-γ when compared between vaccinated and non-vaccinated reactors

- Among the BCG vaccinated tuberculin reactors, the macrophage responses were significantly higher than the BCG vaccinated tuberculin non-reactors.

- There was no significant difference in the responses among the BCG vaccinated tuberculin reactors when compared to the unvaccinated tuberculin reactors. So, tuberculin reactivity may be an indication of new infection and not a marker of BCG.

- The immune responses of unvaccinated tuberculin reactors were significantly higher than the vaccinated tuberculin non-reactors. This indicates that in someway BCG vaccination suppresses the immune response.

These findings support the study-hypothesis that the immune response among the adolescents/young adults is elicited by exposure to mycobacteria and not by the neonatal BCG vaccination. Thus it would seem that BCG given under immunization programme in no way enhances the capacity of the macrophages to overcome natural infection.

This information on the effect of existing neonatal-BCG vaccine and the impact of tuberculin responses on the macrophage host cell may be useful for the development of new and improved TB vaccines. However, these issues should be addressed and followed by large-scale studies to assess its complete mode of action. These findings if confirmed in other studies in future would have a significant relevance for the BCG immunization programme.

- Limitations of the study

- Present study was performed in a small scale level, as the time and other resources were limited.

- In the field analysis part, specific skin test antigens could not be used to delineate the study subjects who were sensitized/infected with the atypical mycobacteria. During the study period, the investigator was unable to get the specific antigens from anywhere. Tuberculins used in the study were those available in Indian market.

- In the immunological experiment part, for technical feasibility *M. tuberculosis* H37RV was alone used as a standard strain in all experiments.

- Only limited number of experiments was carried out to reveal the immune response of the subjects. Role of complement system, Phagosome-Lysosome fusion, MHC markers, Levels of other cytokines etc (using FACS, PCR etc) were not studied because of lack of reagents and other lab facilities.

- Recommendations and Plans for future Work

- Similar kind of studies may be conducted in different study area to provide additional support for the present findings.

- These kinds of studies may be conducted at large scale level at different parts of the world particularly at high TB burden countries to get more information about the influence of BCG vaccination and environmental exposure of mycobacteria on immune response.

- Molecular epidemiological studies will enhance our understanding of different strains involved in TB infection and the immune response against multiple strains in *M. tuberculosis.*

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- Annexure

Annex-I

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI
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Grams : MEDICLAVE, Phone : 22353576 - 79 Fax : 91-44-22353698
Web site : www.tnmmu.ac.in

ETHICAL COMMITTEE CLEARANCE

03/04/06

Title: "Influence of BCG vaccination on the intracellular killing mechanism of human macrophages against Mycobacterium tuberculosis"

The committee members reviewed the above documents and approved.

S.No.	ETHICAL COMMITTEE (EC)	DESIGNATION	SIGNATURES.
1.	Dr.C.V.Bhirmanandam	Vice chancellor IEC chair person	[signature]
2.	Justice Mr. Abdull Hadi	Member	
3.	Dr.C.B.Tharani	Member	[signature]
4.	Dr. D.Jayalakshmi	Member	[signature]
5.	Thiru. M. Vellaisamy	Member	[signature]
6.	Dr. D. Bennet	Member	[signature]
7.	Dr.M.Ananda babu	Member	[signature]
8.	Dr. Annabel Rajasekharan	Member	
9.	Professor N.M.Samuel	Member Secretary	

Annex-II

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY
NO.69, ANNA SALAI, CHENNAI-32, SOUTH INDIA.
DEPARTMENT OF EPIDEMIOLOGY

Influence of BCG vaccination on the intracellular killing mechanism of human monocyte derived macrophages against Mycobacterium tuberculosis

Study Volunteer ID No.______________ Study Area : ___________________ Village __________________ Dist.

Date of Registration ____/____/______

A) Personal Information

1. Name :
2. Gender : 1. Male 2.Female
3. Date Of Birth : ____/____/______
4. Age :
5. Marital status : 1. Married 2. Unmarried 3. Divorcee 4. Widow 5. Others
6. Occupation : 1. Student 2. Govt. 3. Private 4. Landowner 5. Labor 6.Others
7. Address For Communication : ______________________________________

____

____

(B) BCG Scar Status : 1. Seen 2. Not Seen

If seen, size : ___________

(C) Risk factor for TB exposure

8. Family Member with History of TB : 1. Yes 2. No
9. Close contact with a TB patient : 1. Yes 2. No
10. Residence in a refugee group : 1. Yes 2. No
11. Residence in slum : 1. Yes 2. No
12. Health care worker : 1. Yes 2. No
13. International student : 1. Yes 2. No

(D) Risk factor for progression of infection to TB

14. Diabetes : 1. Yes 2. No If yes, duration____________
15. End stage renal disease : 1. Yes 2. No If yes, duration____________
16. Cancer : 1. Yes 2. No If yes, duration____________
17. Asthma : 1. Yes 2. No If yes, duration____________
18. Habits of smoking, Alcoholism or other tobacco use : 1. Yes 2. No

If yes, Habit__________ duration____________

(E) Active TB disease

19. Fever - 1. Yes 2. No If yes, duration____________
20. Cough - 1. Yes 2. No If yes, duration____________
21. Wheezing - 1. Yes 2. No If yes, duration____________
22. Fatigue - 1. Yes 2. No If yes, duration____________
23. Lack of appetite - 1. Yes 2. No If yes, duration____________
24. Sudden weight loss - 1. Yes 2. No

(F) General Health assessment

25. Height ______________ Weight ________________

26. Previous X-ray, CT scan, MRI with abnormal results : 1. Yes 2. No if yes details : ______________

27. Are You Currently on Immunosuppressive Medication
(Steroids, Chemotherapy) : 1. Yes 2. No if yes details : ______________

28. Have You Ever Had A TB Skin Test, : 1. Yes 2. No if Yes, details : ______________

29. Blood Test With Abnormal Results During Past 2 Years : 1. Yes 2. No if yes, details : ______________

(G) Chest X-Ray

1. Recommended 2. Not Recommended

Interpretation

Signature of the Physician

(H)Tuberculin Skin Test

PPD – 1 TU			
Administrated On	Read On	Induration (in mm)	Inference
		Reader 1 ________ Reader 2 ________	

PPD – 10 TU			
Administrated On	Read On	Induration (in mm)	Inference
		Reader 1 ________ Reader 2 ________	

DEPARTMENT OF EPIDEMIOLOGY
THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

NO.69, ANNA SALAI, CHENNAI-32, SOUTH INDIA.

Title of the Research : Influence of BCG vaccination on the intracellular killing mechanism of human monocyte derived macrophages against *Mycobacterium tuberculosis*

Study investigators : C. Mathan Periasamy. MSc.,
Dr. Manjula Datta. MD. DCH. MSc. FRCP.,
Dr. M. Kannapiran. MSc. PhD.,
Dr. V.D. Ramanathan. MBBS. PhD.,

INFORMATION SHEET

The Department of Epidemiology at The Tamilnadu Dr. M.G.R. Medical University has been undertaking research projects with a view of contributing to the development of health policies to the population. One of the studies is " Influence of BCG vaccination on the intracellular killing mechanism of human monocyte derived macrophages against *Mycobacterium tuberculosis* " that includes screening and laboratory diagnosis of tuberculosis and related infections.

Tuberculosis remains a massive global health problem today and India has more number of TB cases than any other country in the world. Every year 1.8 million people in India develop TB. BCG is the only current available vaccine used to prevent TB. But the efficacy of that vaccine in the prevention of TB has shown considerable variations in different populations and trials.

The present study is designed in such a way to give more information on the influence of BCG vaccination on the immune system particularly blood cells with respect to Mantoux response. For this study blood sample from healthy human volunteers is needed.

If you decide to take part in this study, Mantoux test will be performed with PPD's on both forearms. Later 15ml of blood will be drawn from your fore arm vein. Mantoux test is a very simple test used to screen the primary infection with *Mycobacterium tuberculosis*. Moreover this test is widely used in children and adults to diagnose the infection with *Mycobacterium tuberculosis* and related species.

Risks :

1. Mantoux test positive will be indicated by the formation of induration around the site of inoculation.
2. Blood collection may cause pain and swelling at the puncture site.

Side effects :

Since we are not using any drugs or new vaccines there will not be any side effects.

Safety :

1. Physicians and well trained/qualified medical laboratory staffs will collect Blood samples.
2. Efforts will be taken to minimize blood-drawing related problems.
3. Blood collection and Mantoux tests will be carried out by sterile single use disposable syringes with aseptic safety measures.

Note :

- There will be no costs associated with your participation in this study
- Your identity will remain confidential except as required by law
- You are free to withdraw from this study at any time without giving reasons

CONSENT FORM

I have read the information sheet completely and discussed with the study investigators about the procedures and anticipated risks of this present study. I understand that if I need any further information regarding my rights as a subject for this study I may contact the study center.

All the details have been explained to me and I willingly agree to participate in this study and hereby consent to give 15ml blood and to perform Mantoux test.

Signature of the participant

I have completely explained the details of the study to ____________________ . I have answered all questions to the best of my ability.

Signature of the investigator

Signature Witness :

Name & Address :

தமிழ்நாடு டாக்டர் எம்.ஜி.ஆர். மருத்துவப் பல்கலைக் கழகம்

கொள்ளை நோயியியல் துறை

ஆராய்ச்சியின் தலைப்பு	:	பி.சி.ஜி. தடுப்பு ஊசியின் நோய் தடுப்பு செயல்திறன்
ஆய்வாளர்கள்	:	செ. மதன் பெரியசாமி எம்.எஸ்.சி.
		டாக்டர். மஞ்சுளா தத்தா எம்.டி. டி.சி.ஹெச். எம்.எஸ்.சி. எஃப்.ஆர்.சி.பி.
		டாக்டர். கண்ணபிரான் எம்.எஸ்.சி. பி.ஹெச்.டி.
		டாக்டர். இராமநாதன் எம்.பி.பி.எஸ். பி.ஹெச்.டி.

தகவல் படிவம்

எம்.ஜி.ஆர் மருத்துப் பல்கலைக் கழகத்தின் கொள்ளை-நோயியியல் துறையானது மக்களின் சுகாதாரம் மற்றும் நோய்கள் சம்பந்தப்பட்ட பல ஆய்வுகளை மேற்கொண்டுள்ளது. அவற்றுள் பி.சி.ஜி தடுப்பு ஊசியின் பயன்கள் மற்றும் நமது உடலில் அத்தடுப்பூசி ஏற்படுத்தும் நோய் எதிர்ப்புத் தன்மை என்பதும் ஒன்றாகும். இந்தஆய்வுகளில் காச நோயின் ஆரம்ப கட்ட தொற்று நிலையை அதை கண்டறிதலும் அடக்கம்.

காச நோயானது உலக அளவில் இன்று மிகப்பெரிய மக்கள் பிரச்சினையாக உருவெடுத்துள்ளது. ஆண்டுதோறும் சுமார் 18 லட்சம் புதிய காசநோயாளிகள் உருவாகிறார்கள் அதிலும் உலக அரங்கில் இந்தியா காச நோயாளிகள் எண்ணிக்கையில் முதல் இடத்தில் உள்ளது. பி.சி.ஜி. தடுப்பு ஊசி மட்டுமே இந்நோய் வராமல் இருக்க பிறந்தவுடன் போடப்படுகிறது. ஆனால் இந்த தடுப்பு செயல்திறன் பற்றி பல நாடுகளில் மேற்கொள்ளப்பட்ட ஆய்வுகள் பல மாறுபட்ட முடிவுகளை கொடுக்கின்றன.

இந்த ஆராய்ச்சியானது, பி.சி.ஜி. தடுப்பூசியின் செயல்திறன் எந்த அளவுக்கு நமது உடலின் இரத்த நோய் எதிர்ப்பு செல்களில் செயல்படுகிறது என்பதை கண்டறிவதற்காக மேற்கொள்ளப்படுகிறது. எனவே, இந்த ஆராய்ச்சியை இரத்த எதிர்ப்பு செல்களில் செயல்படுத்த வேண்டியுள்ளது.

நீங்கள், இந்த ஆராய்ச்சியில் பங்கு பெறுவது என முடிவு செய்த பின் 'மேன்டோ' எனப்படும் எளிய பரிசோதனை மேற்கொள்ளப்படும். இச்சோதனை காசநோயின் ஆரம்ப கட்ட நிலையை கண்டறிய உதவும். அதன் பின் உங்களிடமிருந்து 15மி.லி இரத்தம் எடுக்கப்படும்.

அபாயங்கள்:-

(1) மேன்டோ பரிசோதனையில் பாசிட்டிவ் முடிவு போடப்பட்ட இடத்தை சுற்றி சிறிய வீக்கமாக தோன்றி வெளிப்படும்.
(2) இரத்தம் எடுக்கப்படும் இடத்தில் வலியோ சிறிய வீக்கமோ தோன்றலாம்.

பக்க விளைவுகள்:-

இந்த ஆராய்ச்சியில் புதிய மருந்துகளோ, தடுப்பூசிகளோ பயன்படுத்தபடாததால் பக்கவிளைவுகள் ஏதும் இல்லை.

முன்னெச்சரிக்கை :-

(1) தகுதி பெற்ற மருத்துவர்கள் மற்றும் அனுபவம் / தகுதி பெற்ற பரிசோதனை அலுவலர்கள் மூலம் இரத்தம் எடுத்துக் கொள்ளப்படும்.
(2) மிகுந்த கவனத்துடன் தவறுகள் ஏற்படாத வண்ணம் இரத்தம் எடுத்துக் கொள்ளப்படும்.
(3) ஆய்விற்கு பயன்படுத்தப்படும் ஊசிகள் அனைத்தும் கிருமி நீக்கப்பட்ட மற்றும் ஒரு முறை மட்டுமே உபயோகிக்க கூடியவை, ஆதலால் நோய் தொற்று ஏற்படாது.

குறிப்பு:-

(1) தங்கள் பங்களிப்பிற்காக பணம் ஏதும் கொடுக்க தேவையில்லை.
(2) தங்கள் அடையாளம் மற்றும் முகவரி ரகசியமாக வைக்கப்படும்.
(3) தங்களுக்கு விருப்பமில்லையெனில் இதிலிருந்து தாமாக விலகிகொள்ளலாம்.

ஒப்புதல் படிவம்

நான் தகவல் படிவத்தை முழுவதுமாக படித்தும், ஆய்வை மேற்கொள்பவரிடம் கலந்தாய்தும் இந்த ஆய்வை பற்றி முழுமையாக தெரிந்து கொண்டேன். மேலும், இந்த ஆய்வை பற்றிய மற்ற தேவையான தகவல்களையும் நான் பெற முடியும் என்றும், எனக்கு விருப்பமில்லையென்றால் நானாகவே எந்த காரணமும் சொல்லாமல் விலகிக் கொள்ளலாம் என்றும் புரிந்து கொண்டேன்.

நான் இந்த ஆராய்ச்சியில் பங்கு கொள்ளவும், மேன்டோ பரிசோதனை மேற்கொள்ளவும் எனது உடலிலிருந்து 15மி.லி. இரத்தம் எடுக்கவும் முழுமையுடன் சம்மதிக்கிறேன்.

இப்படிக்கு

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நான் இந்த ஆய்வில் பங்கு கொள்ள சம்மதித்திருக்கும் திரு. ____________________________ என்பவரிடம் முழு தவகல்களையும் அளித்ததோடு அவரது சந்தேகங்களுக்கான விளக்கத்தையும் முழுமையாக அளித்தேன்.

ஆய்வு மேற்பார்வையாளர்

சாட்சி கையொப்பம்

பெயர் :

முகவரி :

RPMI-1640

1. Dehydrated RPMI media (Gibco, USA) was added to one liter of double distilled water at room temperature and stirred until it gets dissolved.

2. Two grams of sodium bicarbonate (Tissue culture grade, Hi-Media, India) was added to the solution and mixed.

3. The pH of the media was adjusted to 0.2 or 0.3 units lower than the desired pH with 1N HCL / NaOH, as the pH of the media tends to increase during the process of filtration.

4. Media was sterilized by membrane filtration and dispensed into sterile air-tight containers.

5. Storage temperature used for the media was 2 – 4 ^{0} C.

Middlebrook 7H9 broth

1. Appropriate volume of Middlebrook 7H9 broth base (BD, USA) was weighed and added to 900 ml of double distilled water and stirred until it gets dissolved.

2. Two ml of glycerol was added and the media-base was autoclaved at 121^{0}C for 15 minutes.

3. After autoclaving, solution was allowed to cool and 100 ml of ADC (BD, USA) was added and mixed thoroughly.

4. Media was dispensed into sterile air-tight containers under aseptic conditions.

Middlebrook 7H11 agar

1. Appropriate volume of Middlebrook 7H11 agar base (BD, USA) was weighed and added to 900 ml of double distilled water and stirred until it gets dissolved.

2. Two ml of glycerol was added and the media-base was autoclaved at 121^0C for 15 minutes.

3. After autoclaving, solution was allowed to hand-bearable heat and 100 ml of OADC (BD, USA) was added thoroughly.

4. Media was poured into sterile Petri-plates under aseptic conditions and allowed to solidify.

Reagents for Superoxide Dismutase assay

- Reagent R1 – 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo(c) fluorene
- Reagent R2 – 1-methyl-2-vinylpyridinium
- Buffer – 2 – amino-2-methyl-1,3-propanediol
  (Calbiochem – Cat no. 574600, Germany)

Reagents for Glutathione assay

- Reagent R1 – Solution of chromogenic reagent in HCL
- Reagent R2 – 30% NaOH
- Buffer (Solution 3) Potassium phosphate containing diethylenetriamine pentaacetic acid (DTPA) and lubrol

  (Calbiochem – Cat no. 354102, Germany)

Reagents for Tumour Necrosis Factor-α Assay

Capture Antibody – Anti-human TNF monoclonal antibody

Detection Antibody – Biotinylated anti-human TNF monoclonal antibody

Enzyme Reagent – Avidin-horse radish peroxidase conjugate

Substrate Solution – Tetramethylbenzidine (TMB) and Hydrogen Peroxide

Standards – Recombinant human TNF, lyophilized

(BD – Cat no. 555212, USA)

Reagents for Interferron-γ Assay

Capture Antibody – Anti-human IFN- γ monoclonal antibody

Detection Antibody – Biotinylated anti-human IFN- γ monoclonal antibody

Enzyme Reagent – Avidin-horse radish peroxidase conjugate

Substrate Solution – Tetramethylbenzidine (TMB) and Hydrogen Peroxide

Standards – Recombinant human IFN-γ, lyophilized

(BD – Cat no. 555142, USA)

ELISA Buffers and solutions

Coating Buffer (0.1 M Sodium Carbonate) pH 9.5

$NaHCO_3$ – 8.40 g

Na_2CO_3 – 3.56 g

Dis. Water – 1 liter

Phosphate – Buffered Saline, pH 7.0

NaCl – 8.0 g

Na_2HPO_4 – 1.16 g

KH_2PO_4 – 0.2 g

KCl – 0.2 g

Dis. Water – 1 liter

Assay Diluent

PBS with 10% Fetal Bovine Serum-heat inactivated, pH 7.0

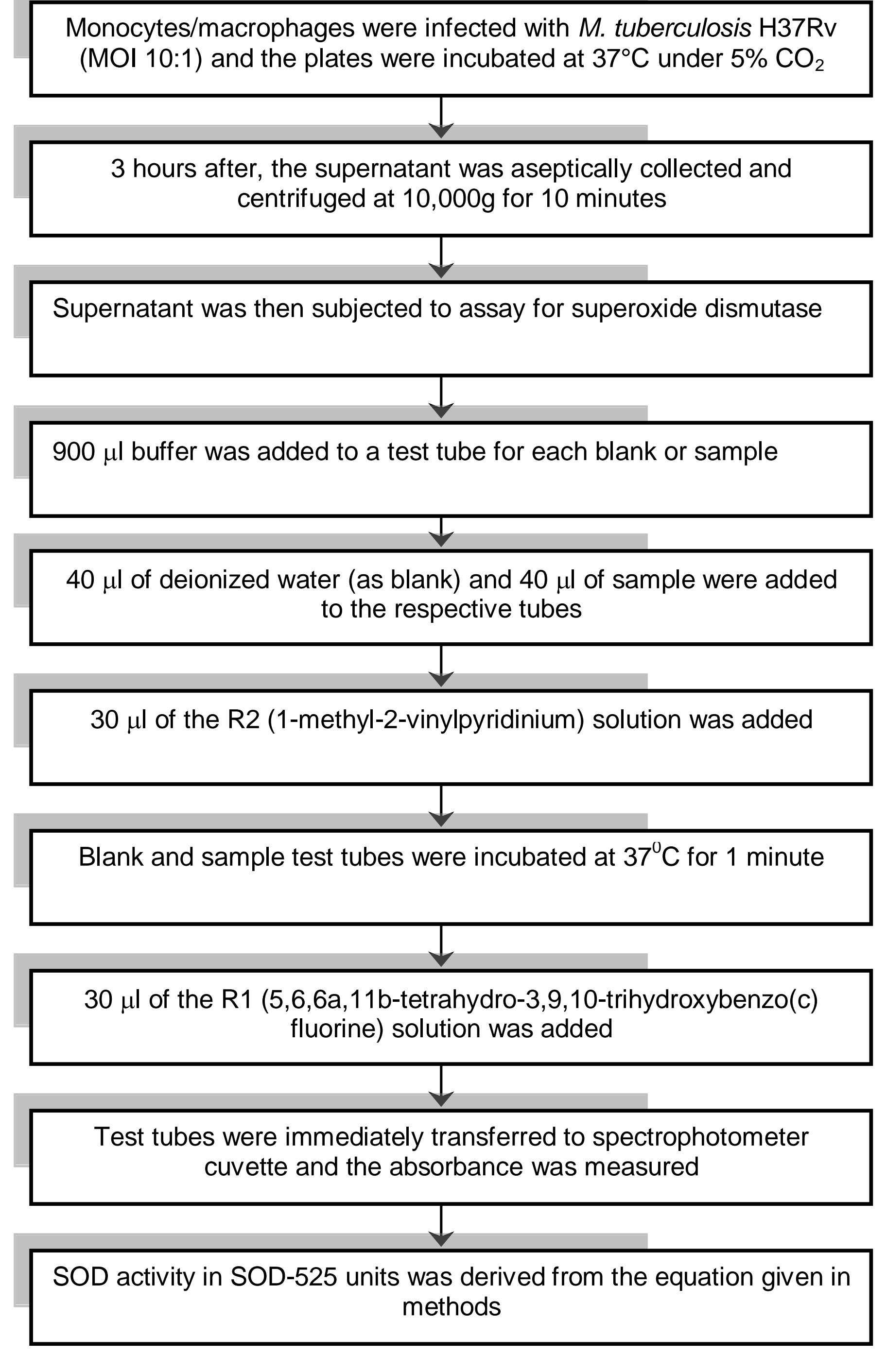
Wash Buffer

PBS with 0.05% Tween-20

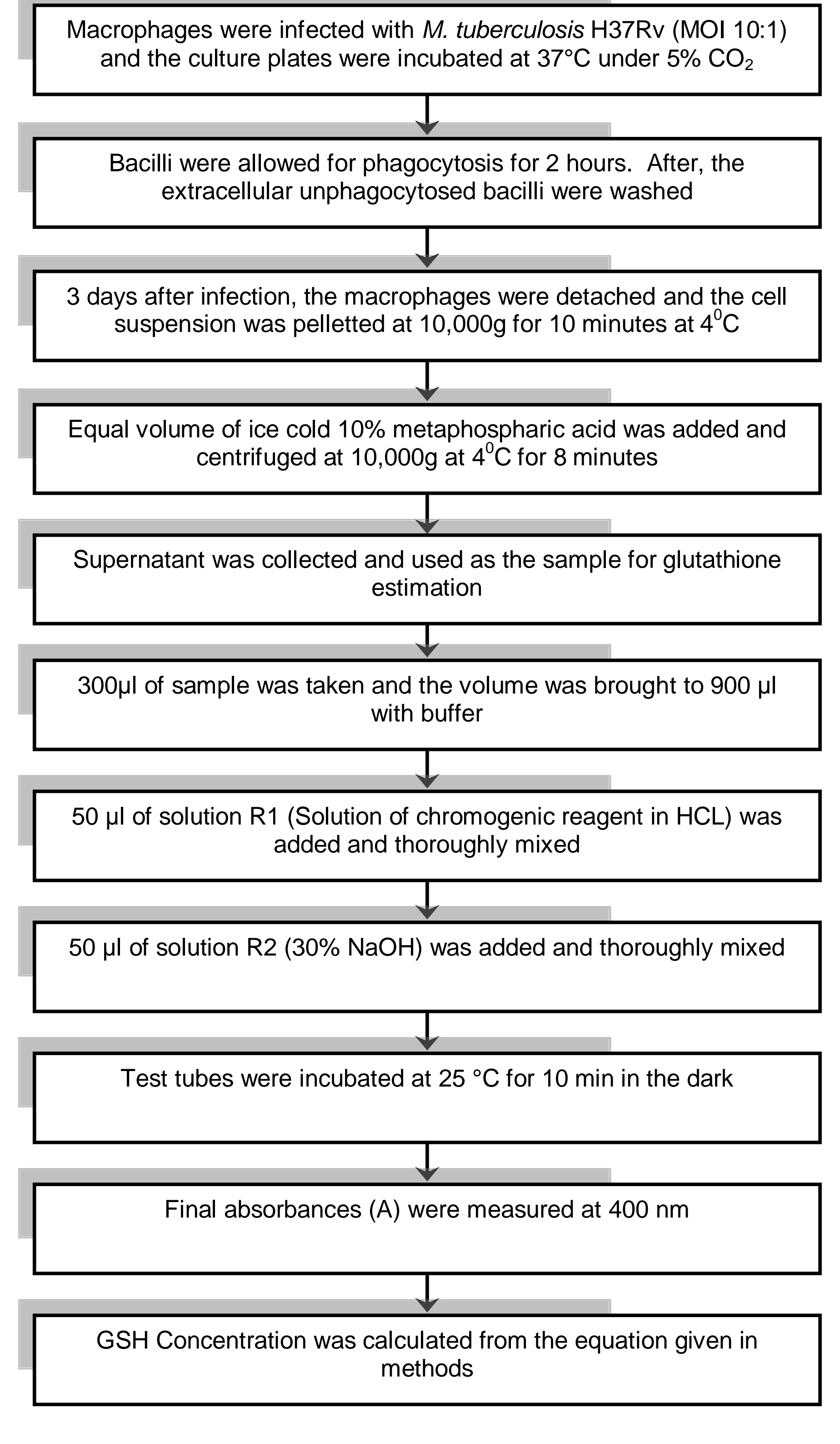
Stop Solution

2 N H_2SO_4

Superoxide Dismutase assay



Glutathione assay



Cytokine Estimation – ELISA

Heparinised blood was diluted 1 in 3 with RPMI-1640 followed by the infection of *M. tuberculosis* H37Rv at 10^5 CFU per well

↓

Plates were incubated at 37°C under 5% CO_2 (24 hr for TNF-alpha; 96 hr for IFN-gamma)

↓

After infection, the samples were centrifuged at 10000g for 10 minutes. The supernatants were collected and stored at – 80^0C

↓

ELISA plate wells were coated with 100 µl per well of capture antibody diluted in coating buffer. The plates were sealed and incubated overnight at 4^0C

↓

Wells were aspirated and washed 3 times with wash buffer

↓

Plates were blocked with 200 µl/well assay diluent by incubating at RT for 1 hour. Wells were aspirated and washed again with wash buffer for three times

↓

100 µl of each standard, sample and control were added into appropriate wells. Plates were sealed and incubated overnight at 4^0C

↓

Wells were aspirated and washed again five times with wash buffer

↓

100 µl of working detector was added to each well. Plates were sealed and incubated for 1 hour at RT

↓

Wells were aspirated and washed again seven times with wash buffer

↓

100 µl of substrate solution was added to each well. Plates were incubated (without plate sealer) for 30 minutes at room temperature in the dark

↓

50 µl of stop solution was added to each well. Within 30 minutes of stopping reaction the absorbance was read at 450nm

↓

Samples were assayed from the standard curve using the software Softmaxpro (Molecular devices, USA).