

**IMMUNOLOGICAL STUDIES OF DNA (pJWVacII)  
AND SURFACE DISPLAY (r-STVacII) VACCINE  
CANDIDATES EXPRESSING A SYNTHETIC  
MULTIEPITOPE GENE OF *MYCOBACTERIUM  
TUBERCULOSIS* IN A PRIME BOOST STRATEGY  
USING A MOUSE MODEL**

**NORHANANI BT. MOHD REDZWAN**

**UNIVERSITI SAINS MALAYSIA**

**2008**



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**by**

**NORHANANI BT. MOHD REDZWAN**

**Thesis submitted in fulfillment of the  
requirement for the Degree of  
Master of Science**

**2008December  
September 2007**

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## ACKNOWLEDGEMENTS

In the name of Allah, the most Generous and the most Merciful. All praise is due to Allah, for giving me inspiration and stoutheartedness along this journey.

During this research project, there are several people involved directly or indirectly whom I wish to acknowledge in this section.

I wish to thank my supervisor, Assoc. Prof. Mustaffa Musa, for his support, excellent guidance and supervision throughout the experimental work, research investigations and also for providing all the necessary facilities to carry out this study. His guidance is greatly appreciated.

I would like also to thank my Co-supervisor, Prof. Zainul F. Zainuddin for his kind guidance, overall comments and helpful discussions during this study. Thanks also to Mr. Jamaruddin Mat Asan from PPSK and Mrs. Melisa from the Immunology Laboratory for technical assistance in doing some experimental work.

Special thanks to my friends and colleagues in the laboratory especially Azura, Ayuni, Tini, Bad, Eza, Nurul, Abdah and Kak Salwana. In addition, I would like to thank my friends in the NMN and SS research groups for both friendship and assistance during the course of this study.

Not to forget, Dr. Nurul Khaiza, Dr. Noor Suryani, Dr. Che Maraina, Dr. Wan Zuraida and all staff in Immunology department for their support. To all my friends outside the laboratory, thanks for being there and encouraging me when needed. I hope you feel my gratitude.

My deepest appreciation will be to my family especially my beloved parents for their great support, patience, love, encouragement and providing me with the inspiration to pursue my study.

May Allah (s.w.t)God bless you all, Amieen.

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## LIST OF ABBREVIATIONS

AFB	Acid fast bacillus
$\alpha\beta$	Alpha beta
Ag85	Antigen 85
APCs	Antigen presenting cells
BCG	Bacille Calmette Guerin
CMI	Cell mediated immunity
CFU	Colony forming unit
CTL	Cytotoxic T lymphocyte
ddH <sub>2</sub> O	Deionised distilled water
DTH	Delayed type hypersensitivity
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
$\gamma\delta$	Gamma delta
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
i.m	Intra <del>m</del> uscular
i.p	Intraperitoneal
kDa	kilodalton
LB	Luria- <del>B</del> ertani
MHC	Major histocompatibility complex
mAbs	Monoclonal antibodies
MDR-TB	Multi- <del>d</del> rug resistant TB
NAA	Nucleic acid amplification
NK	Natural killer

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Nramp	Natural-resistance-associated macrophage protein
O.D	Optical density
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPD	Purified protein derivative
rBCG	Recombinant bacilli Calmette Guerin
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RD	Region of difference
RE	Restriction enzyme
SIV	Simian immunodeficiency virus
SI	Stimulation index
Th	T helper
TAP	Transporter associated protein
TB	Tuberculosis
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UV	Ultraviolet
WHO	World Health Organization

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**KAJIAN IMUNOLOGI CALON VAKSIN DNA (pJWVacII) DAN VAKSIN  
'SURFACE DISPLAY' (r-STVacII) YANG MENGEKSPRESKAN GEN  
MULTIEPITOP SINTETIK MYCOBACTERUM TUBERCULOSIS DALAM  
STRATEGI 'PRIME BOOST' MENGGUNAKAN MODEL MENCIT.**

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**ABSTRAK**

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Tuberculosis (TB) pada manusia adalah disebabkan oleh patogen *Mycobacterium tuberculosis* dan merupakan salah satu penyakit utama di dunia. Satu-satunya vaksin TB yang terdapat pada masa ini ialah strain *M. bovis* yang telah dilemahkan iaitu, Bacille Calmette Guerin (BCG). Bagaimanapun, efikasi perlindungan BCG merangkumi julat 0 ke 80% di tempat kajian yang berlainan. Kesan perlindungan BCG adalah ketara pada kanak-kanak tetapi tidak menunjukkan perlindungan terhadap TB paru-paru di kalangan orang dewasa yang menjadi masalah utama. Vaksin DNA merupakan salah satu cara baru untuk mengawal penyakit berjangkit dan boleh merangsang tindak balas kedua-dua sel humoral dan selular. Hasil kajian lepas, calon vaksin pJWVacII dan r-STVacII telah digunakan dalam kajian ini dengan menggunakan strategi 'prime-boost'. Vaksin DNA, pJWVacII telah diberikan secara intraotot kepada mencit manakala vaksin 'surface display' pula telah diberikan secara oral kepada mencit. Splenosit dari mencit yang diimmunisasi telah diuji dengan pelbagai ujian keimunan. Keputusan menunjukkan bahawa splenosit dari mencit yang diimmunisasi memberikan peningkatan gerak balas proliferasi apabila dirangsang dengan antigen (Inak-nVacII). Analisis sitokin intrasel ke atas splenosit juga menunjukkan kedua-dua CD4<sup>+</sup> dan CD8<sup>+</sup> sel T menghasilkan IL-2 dan IFN- $\gamma$  berikutan rangsangan antigen. Dalam kaedah 'prime-boost', kajian menunjukkan kaedah 'prime' dengan pJWVacII dan 'boost'

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dengan r-STVaciI adalah strategi terbaik untuk merangsang respon keimunan dalam mencit. Sebagai kesimpulan, data yang diperoleh dari kajian ini mencadangkan bahawa vaksin DNA digabungkan dengan vaksin '*surface display*' menggunakan kaedah '*prime-boost*' merupakan salah satu strategi baru untuk membangunkan calon vaksin terhadap TB.

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BOOST STRATEGY USING A MOUSE MODEL**

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**ABSTRACT**

Tuberculosis (TB) in humans is caused by the bacterial pathogen *Mycobacterium tuberculosis* and is still one of the major health problems worldwide. The only TB vaccine currently available is an attenuated strain of *M. bovis*; Bbacillei Calmette Guerin (BCG). BCG demonstrated variable protective efficacies ranging from 0 to 80% in different field trials. In addition, BCG prevents is effective at preventing childhood manifestation of TB but it does not prevent the most prevalent disease from which is pulmonary TB in adults. DNA vaccination is an new important new approach to the control of infectious agents and induces both humoral and cellular immune responses. Two pPreviously constructed vaccine candidates, pJWVaciII and r-STVaciII were used in this study applying employing a prime-boost strategy. The nNaked DNA vaccine, pJWVaciII was given intramuscularly to mice whilst thee surface display vaccine, r-STVaciII was given orally to mice. Splenocytes from the vaccinated mice were tested for various immunological tests. The results showed that splenocytes from immunized mice were found to proliferate more aggressively when stimulated with the antigen (Inak-nr-VaciII). Flow cytometric intracellular cytokine analysis of splenocytes from vaccinated mice also showed that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells produce IL-2 and IFN- $\gamma$  following stimulation with the antigens. In the prime-boosting approach, the study was showed that mice

primed with the naked DNA vaccine, pJWVacII and boosted with the surface display vaccine, r-STVacII is the best strategy to stimulate immune response in mice. As a conclusion, the data obtained from this study suggest that DNA vaccination in combination with surface display vaccination using prime-boost approach provides a new strategy for developing a candidate vaccine against TB.

### ABSTRAK

~~———— Tuberculosis (TB) pada manusia adalah disebabkan oleh patogen bakteria *Mycobacterium tuberculosis* dan merupakan salah satu penyakit utama di dunia. Satu-satunya vaksin TB yang terdapat pada masa ini ialah strain *M. bovis* yang telah dilemahkan iaitu, *M. bovis* Bacille Calmette Guerin (BCG). Bagaimanapun, efikasi perlindungan BCG adalah berlainan darimerangkumi julat 0 ke 80% di tempat kajian yang berlainan. Tambahan pula, Kkesan perlindungan BCG adalah ketara pada kanak-kanak tetapi tidak menunjukkan perlindungan terhadap TB paru-paru di kalangan orang dewasa yang menjadi masalah utama. Vaksin DNA merupakan salah satu cara baru untuk mengawal ejen infeksipenyakit berjangkit dan boleh merangsang tindak balas kedua-dua sel humoral dan selular. Hasil kajian lepas, calon vaksin pJWVacII dan r-STVacII telah digunakan dalam kajian ini dengan menggunakan strategi 'prime boost'. Vaksin DNA, pJWVacII telah diberikan secara intraotot kepada mencit manakala vaksin 'surface display' pula telah diberikan secara oral kepada mencit. Splenosit dari mencit yang diimmunisasi telah diuji dengan pelbagai ujian keimunan. Keputusan menunjukkan bahawa splenosit dari mencit yang diimmunisasi memberikan peningkatan gerak balas proliferasi apabila dirangsang dengan antigen (rVacII). Analisis sitokin~~

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intrasel ke atas splenosit juga menunjukkan kedua-dua CD4<sup>+</sup> dan CD8<sup>+</sup> sel T menghasilkan IL-2 dan IFN- $\gamma$  berikutan rangsangan antigen. Dalam kaedah 'prime-boost', kajian menunjukkan kaedah 'prime' dengan pJWVaeII dan 'boost' dengan r-STVaeII adalah strategi terbaik untuk merangsang respon keimunan dalam miceit. Sebagai kesimpulan, data yang diperolehi dari kajian ini mencadangkan bahawa vaksin DNA digabungkan dengan vaksin 'surface display' menggunakan kaedah 'prime-boost' merupakan salah satu strategi baru untuk membangunkan calon vaksin terhadap TB.

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Introduction to TB

Tuberculosis (TB) is a contagious and potentially fatal disease that can affect almost any part of the body but manifests mainly as an infection of the lungs. It is caused by a bacterial microorganism, the tubercle bacillus or *Mycobacterium tuberculosis*. TB infection can either be acute and short-lived or chronic and long-term. Approximately 1.7 billion people or one-third of the world's population are infected with the predominant TB organism, *M. tuberculosis*. Currently, 10 to 15 million people in the United States have latent TB infection, and 10 percent of them will develop active disease at some point in their lives (Diana, 2000).

Most people infected with *M. tuberculosis* never develop active TB. However, in people with weakened immune systems, especially those infected with the human immunodeficiency virus (HIV), TB organisms may overcome the body's defenses, multiply, and cause active disease (Ellner, 1990). Each year, 8 million people worldwide develop active TB and 3 million die. TB is often one of the first secondary infections to be activated in HIV – positive individuals. Moreover, poverty, malnutrition and their contributing factors such as political disorganization and war also contribute to the increase rate of TB. Figure 1.1 shows TB notification rate around the world.

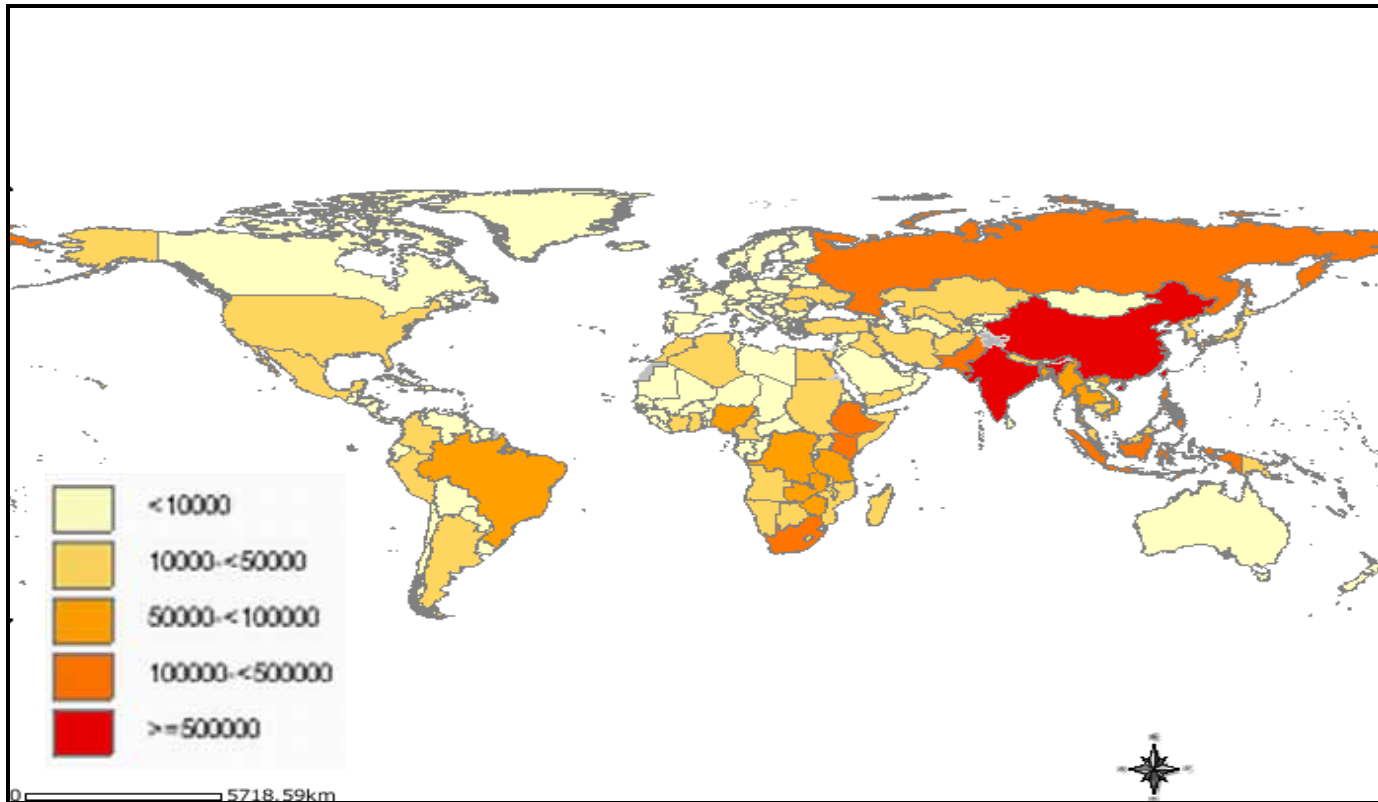


Figure 1.1: Tuberculosis notification rates, as of 22 March 2006 (Source: WHO Stop TB Department, website: [www.who.int/tb](http://www.who.int/tb))

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## 1.2 History of TB

In the second half of the 17th century, when TB caused high levels of death rates in Europe, John Bunyan gave the title 'captain of all these men of death' for TB, which was also known as the white plaque. Pulmonary TB was known since the time of Hippocrates as phthisis, derived from the Greek for 'wasting away'. In 1680, the French, Franciscus Sylvius carried out anatomic-pathologic studies in pulmonary nodules from TB patients, which he named as 'tubercula' (small knots). These knots were believed to be only some type of tumor or abnormal gland. In 1722, a British doctor Benjamin Marten proposed that TB could be transmitted through the 'breath' of a sick person (reviewed by Rodrigo *et al.*, 2006).

In 1882, Robert Koch isolated and cultured *M. tuberculosis* from crushed tubercles and identified the bacterium as the TB etiological agent. In 1896, an American bacteriologist demonstrated that bovine TB was not caused by *M. tuberculosis* but ~~by a closely related bacterium~~ by a closely related bacterium, *M. bovis*. Twelve years later, Albert Calmette and Camille Guerin isolated the bovine variant from its host and grew the bacilli in dispersed culture containing ox bile. After 13 years of experimentation, the variant was administered for the first time in humans orally, as an attempt to immunize a child whose mother died in childbirth as a victim of TB.

However, WHO indicated that there have not been great effects on the global problem since the time of Koch (Bloom & Murray, 1992). Currently, TB causes



more human deaths than any other single infectious agent, standing for 26% of all preventable deaths and 7% of all deaths (reviewed by Rodrigo *et al.*, 2006).

### 1.3 *Mycobacterium tuberculosis*

The TB bacterium is a rod-shaped bacterium, non-motile, non-spore forming, 1-4  $\mu\text{m}$  in length, and between 0.3-0.6  $\mu\text{m}$  in diameter, making them smaller than most bacterial pathogens (Iseman, 2000 and Akemi *et al.*, 2003).

This bacterium ~~belonging~~ belongs to the family Mycobacteriaceae and the order Actinomycetales. *M. tuberculosis*, like other mycobacteria, has an unusual cell wall, a waxy coat comprised of fatty molecules whose structure and function are not well known. This cell wall appears to allow *M. tuberculosis* to survive in its preferred environment: inside immune cells called macrophages, which ordinarily degrade pathogens with enzymes. The coat of *M. tuberculosis* also renders it impermeable ~~ility~~ to many common drugs.

*M. tuberculosis* and other mycobacteria are also called as “acid fast” bacteria (AFB) which means that they retain certain dyes following an acid-alcohol decolorization step and this characteristic is related to the complex cell wall structure that contains derivatives of mycolic acid (Floyd *et al.*, 1992). In tThe most common staining technique, Ziehl-Neelsen stain, AFB ~~is~~ are stained a bright red, which stands out clearly against a blue background. It ~~is also~~ can also be visualized by fluorescent microscopy and by auramine-rhodamine stain (Batzing, 2002).

There are several factors that contribute to the difficulty ~~of in the~~ study of *M. tuberculosis* in the laboratory. First, the bacteria multiply very slowly, only once every 24 hours and take a month to form a colony. Two media are often used to grow *M. tuberculosis*: Middlebrook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium (American Thoracic Society, 2000).

In comparison, other organism such as *E. coli* form colonies within eight hours. Moreover, TB bacilli tend to form clumps which are difficult to work with or to count ~~them~~the cells. Most daunting, *M. tuberculosis* is a dangerous, airborne organism that can be studied only in laboratories that have specialized safety equipment.

Several species of mycobacteria with similar growth characteristics and biochemical reactions are classified together ~~into as the~~ *M. tuberculosis* complex (Cole, 2002). This complex includes *M. bovis*, *M. africanum* and *M. microti* which can also causes TB in mammals. The first two are very rare causes of disease and the last one do not cause human disease (Brosch *et al.*, 2000).

### 1.3.1 The cell wall of *M. tuberculosis*

Mycobacteria produce an extremely uncommon cell wall structure. It is composed of a multilayered cell envelope which basically consists a multilayered cell envelope which basically consists of (-from inside the cells to the outer surface): a plasma membrane and three covalently associated macromolecules

such as peptidoglycan, arabinogalactan and mycolic acid or glycolipids (Figure 1.2). The plasma membrane is composed of ~~a thicker outer leaflet and a thinner inner leaflet. The p~~Plasma membrane was observed ~~is thought to be to be~~ a smooth, asymmetrical stained structure closely associated with the peptidoglycan layer, with a narrow constant space between them (Akemi *et al.*, 2003).

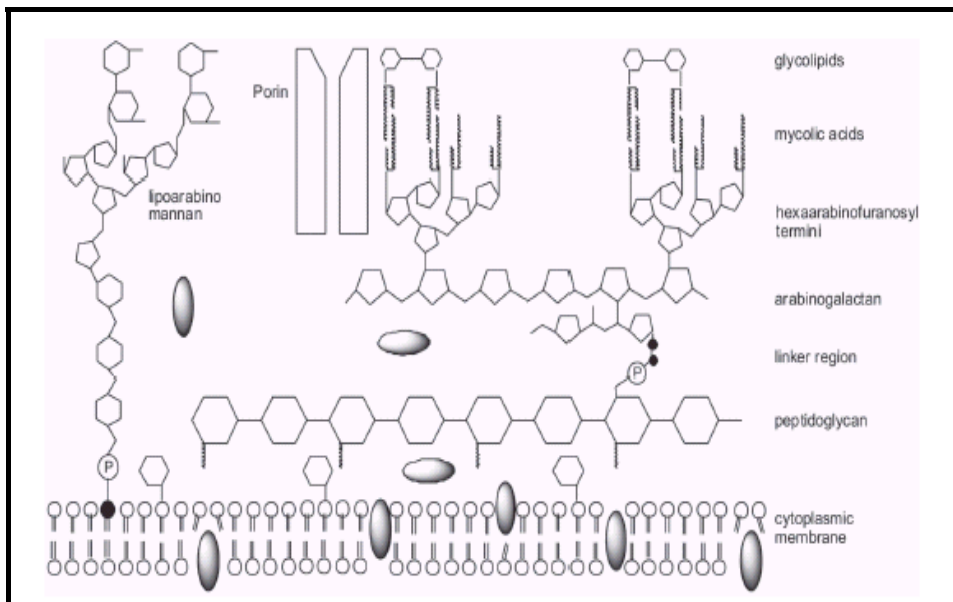


Figure 1.2: Schematic representation of the mycobacterial cell wall (Adapted from Rodrigo *et al.*, 2006)

a thicker outer leaflet and a thinner inner leaflet. The plasma membrane is thought to be a smooth, asymmetrical stained structure closely associated with the peptidoglycan layer, with a narrow constant space between them (Akemi *et al.*, 2003).

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The peptidoglycan layer tangentially intrudes into the cytoplasm and formed a septal wall together with the plasma membrane. The width of the peptidoglycan layer of *M. tuberculosis* was measured to be approximately 10-15 nm (Akemi *et al.*, 2003) and contains *N*-glycolylmuramic acid instead of the usual *N*-acetylmuramic acid, which is found amongst most other bacteria.

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From the mycobacterial cell envelope study carried out by Rastogi (1991) show the presence of an arabinogalactan layer on the peptidoglycan which is attached by phosphodiester links. This compound is supposed to be richly found in the cell wall of *M. tuberculosis* and to be responsible for the thickening of the cell wall (Akemi *et al.*, 2003). Approximately

10% of the arabinose residues in the arabinogalactan are substituted by mycolic acids (Mc-Neil & Brennan, 1991).

Mycolic acids are branched fatty acids that have a short and a long branch with 22 to 24 and 40 to 64 carbons respectively (Jarlier & Nikaido, 1994). The cell wall also contains several other free lipid species, which are not covalently attached to this basal skeleton (the mycolylarabinogalactan-peptidoglycan complex). These lipids can act as antigens in the host (Brennan & Nikaido, 1995). In the arabinogalactan polysaccharide, both galactan main chain and arabinan side branches are designed-structured in such a manner that would ensures maximum mobility between sugar residues. The mycolic acid residues are esterified to approximately two-thirds of the non-reducing termini of this highly branched polysaccharide (Mc Neil & Brennan, 1991).

Furthermore, the mycobacterial cell wall fluidity gradient appears to have an opposite orientation compared to all Gram-negative bacteria as the more external regions are more fluid than the internal ones. Amongst mycobacterial species, *M. tuberculosis* is one of the most permeable to hydrophilic antimycobacterial agents and thereby, less resistant to such drugs (for instance, etham-butol) (Brennan & Nikaido, 1995).

Since mycobacteria are relatively resistant tee to drying, alkali and many other chemical disinfectants, it is thus very difficult to prevent *M. tuberculosis* transmission in urban institution environments. This resistance to therapeutic such external agents is basically conferred by the extremely uncommon

mycobacterial cell wall structure (Brennan & Nikaido, 1995). The unusual cell wall also permits the microorganism to survive inside ~~the macrophages which~~ that would usually destroy phagocytosed pathogens (NSB Editorial Comment, 2000).

### 1.3.2 Genome of *M. tuberculosis*

*M. tuberculosis* has a circular chromosome ~~with of~~ with 4,411,529 base pairs with a 65.6% G+C content. Although the *M. tuberculosis* genome is smaller than that of *E. coli*, it is very versatile, coding for most of the typical bacterial anabolic and catabolic pathways and amino acid synthesis/degradation (Cole *et al.*, 1998). The feature that differentiates *M. tuberculosis* from any other bacteria is the presence of a genome with approximately 4000 genes, mostly coding for enzymes involved in lipogenesis lipolysis ~~(for bacterial survival insides its host)~~ and lipolysis lipogenesis ~~(for cellular envelope synthesis and turnover)~~. There ~~have been at~~ least 2 prophages have been detected in its genome.

Analyses of the genome suggest that this pathogen may be able to alter protein expression patterns thus presenting to its host's immune system as a moving target and therefore interfering in protective immunological responses, which can be involved with the fact that *M. tuberculosis* has a low lysis level in culture. ~~Through protein expression pattern alteration, these pathogens can be presented to their host's immune system as a moving target, interfering in the immunological response by antigen processing inhibition. Thereby, ensuring a greater survival probability to the bacteria (Cole *et al.*, 1998).~~

#### 1.4 Transmission of TB

TB is primarily an airborne disease. The disease is not likely to be transmitted through personal items belonging to those with TB, such as clothing, bedding or other items they have touched. TB is spread from person to person in droplets nuclei expelled from the lungs when a TB sufferer coughs, sneezes, sings or laughs. Adequate ventilation is the most important measure to prevent the transmission of TB. This is because most infected people expel relatively few bacilli and, transmission of TB usually occurs only after prolonged exposure to someone with active TB.

Most people are likely to be contagious when their sputum contains bacilli. This happens when they cough frequently and when the extent of their lung disease as revealed by a chest x-ray, is great. Only people with active disease are contagious. Droplet nuclei are tiny and remain in the air for prolonged periods, ready to be inhaled. They are small enough to bypass the natural defenses of upper respiratory passages such as hairs in the nose or the hair like cilia in the bronchial tubes. Infection begins when the bacilli reaches the tiny air sacs of the lungs known as alveoli, where they multiply within macrophages (Schluger & Rom, 1998 and, Frieden *et al.*, 2003). People who have been treated with appropriate drugs for at least two weeks usually will not become infectious.

There are three factors that influence transmission of *M. tuberculosis*. First is the number of viable bacilli in patient's sputum and their concentration in the air. Secondly, the length of time an exposed person breathes the contaminated air and thirdly, the immune status of the exposed individuals (Horsburgh, 1996).

## **1.5 Pulmonary and extrapulmonary TB**

### **1.5.1 Pulmonary TB**

Pulmonary TB is TB that affects the lungs, and represents about 85% of newly diagnosed cases ~~diagnosed~~. It usually presents with a cough, which may or may not produce sputum. In time, more sputum is produced that is streaked with blood. The cough may be present for weeks or months and may be accompanied by chest pain and shortness of breath. Persons with pulmonary TB often run a low-grade fever and suffer from night-sweats. The patient often loses interest in food and may lose weight. If the infection allows air to escape from the lungs into the chest cavity (pneumothorax) or if fluid collects in the pleural space (pleural effusion), the patient may have difficulty breathing. The TB bacilli may travel from the lungs to lymph nodes in the sides and back of the neck. Infection in these areas can break through the skin and discharge pus.

### **1.5.2. Extrapulmonary TB**

Although the lungs are the major sites of damage caused by TB, many other organs and tissues in the body may be affected. About 15% of newly diagnosed cases of TB are extrapulmonary, with a higher proportion of these being HIV-infected persons. The usual progression of the disease is to begin in the lungs (Figure 1.3) and spread to locations outside the lungs. In some cases, however, the first sign of disease appears outside the lungs.



Tissues or organs that may be affected by TB include bones, kidneys, female reproductive organs, abdominal cavity, joints and meninges. All parts of the body such as skin, intestines, adrenal glands and blood vessels can be also infected by *M. tuberculosis* (Bloom and Murray, 1992).

Miliary TB is a life-threatening condition that occurs when large numbers of tubercle bacilli spread throughout the body. Huge numbers of tiny tubercular lesions develop that cause marked weakness and weight loss, severe anemia and gradual wasting of the body.

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## 1.6 Symptoms of TB

TB bacteria become active if the immune system cannot stop them from growing. The active bacteria begin to multiply in the body and cause active TB disease. The bacteria attack the body and destroy tissues. If this occurs in the lungs, the bacteria can actually create a hole in the lung. Some people develop active TB disease soon after becoming infected, before their immune system can fight the TB bacteria. Other people may get sick later, when their immune system becomes weak.

In most people who breathe in an environment with containing the TB bacteria and become infected, the body is able to fight the bacteria to stop them from growing. The bacteria become inactive, but they remain alive in the body and can become active later. This is called latent TB infection. Many people who have latent TB infection never develop active TB disease. In these people, the TB bacteria

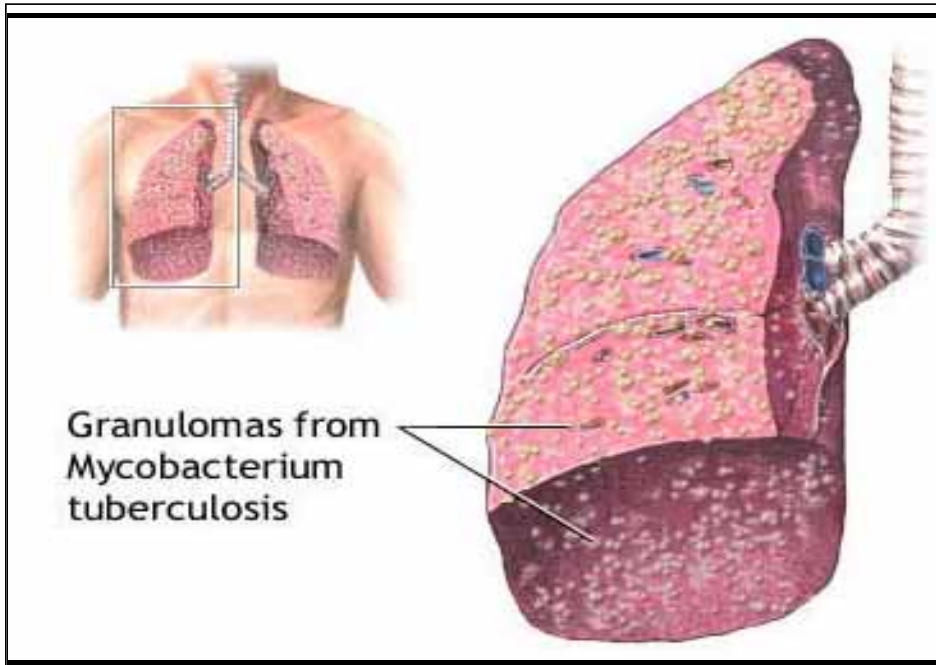


Figure 1.3: Progression of TB in the lung. Granulomas from *Mycobacterium tuberculosis* will spread to locations outside the lungs or called as extrapulmonary TB (Adapted from ADAM Health Illustrated Encyclopedia, 2006).

Granulomas from  
*M. tuberculosis*

can fight the TB bacteria. Other people may get sick later, when their immune system becomes weak.

In most people who breathe in an environment containing the TB bacteria and become infected, the body is able to fight the bacteria to stop them from growing. The bacteria become inactive, but they remain alive in the body and can become active later. This is called latent TB infection. Many people who have latent TB infection never develop active TB disease.

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In these people, the TB bacteria remain inactive for a lifetime without causing disease. However, in other people, especially people who have weak immune systems, the bacteria become active and cause TB disease. Table 1.1 shows the differences between latent TB infection and active TB disease.

## 1.7 Immune response to TB

*M. tuberculosis* is a very successful pathogen that can survive and persist in the human host in the face of a robust immune response. Although the organism can multiply within the macrophage, it can still be inhibited and killed when the macrophage is activated. There are two antimycobacterial mechanisms of in macrophages that can result in destruction of mycobacteria. First, macrophages can produce nitric oxide and related reactive nitrogen intermediates (RNIs) via the action of the form of nitric oxide synthase (NOS2). Following RNI and NOS2 production, IFN- $\gamma$  and TNF- $\alpha$  were induced as an antimycobacterial activity (Flynn, 2004).

~~Another antimycobacterial mechanism of macrophage is phagolysosome fusion in which which contains phagosomes containing ingested bacteria fuses with lysosomes containing degradative enzymes and other antibacterial substances. and This is a primary mechanism by which macrophages control infection. This fusion will increase when the macrophage is activated by IFN  $\gamma$  or other cytokines. Unfortunately, *M. tuberculosis* that is initially within a phagosome, then can also inhibit phagolysosome fusion and acidification in “non activated” macrophages. Therefore, the mycobacteria can avoid being killed by the macrophages.~~

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Table 1.1: The differences between latent TB infection and active TB disease (National Adapted from Centers for HIV/AIDS, Viral Hepatitis, STD and TB Prevention Disease Control & Prevention, 2007)

A Person with Latent TB Infection	A Person with Active TB Disease
<ul style="list-style-type: none"> <li>Has no symptoms</li> </ul>	<ul style="list-style-type: none"> <li>Has symptoms that may</li> </ul>

<ul style="list-style-type: none"> <li>• Does not feel sick</li> <li>• Cannot spread TB to others</li> <li>• Usually has a positive skin test or QuantiFERON-TB<sup>®</sup> Gold test</li> <li>• Has a normal chest x-ray and sputum test</li> </ul>	<p>include:</p> <ul style="list-style-type: none"> <li>• a bad cough that lasts 3 weeks or longer</li> <li>• pain in the chest</li> <li>• coughing up blood or sputum</li> <li>• weakness or fatigue</li> <li>• weight loss</li> <li>• no appetite</li> <li>• chills</li> <li>• fever</li> <li>• sweating at night</li> </ul> <ul style="list-style-type: none"> <li>• May spread TB to others</li> <li>• Usually has a positive skin test or QuantiFERON<sup>®</sup>-TB Gold test</li> <li>• May have an abnormal chest x-ray, or positive sputum smear or culture</li> </ul>
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Another antibacterial mechanism of macrophage is phagolysome fusion in which phagosomes containing ingested bacteria fuses with lysosomes containing degradative enzymes and other antibacterial substances. This is a primary mechanism by which macrophages control infection. Fusion will

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inhibit phagolysome fusion and acidification in “non-activated” macrophages.  
Therefore, the mycobacteria can avoid being killed by the macrophages.

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### 1.7.1 Early host response

The site of initial infection is usually the alveoli, the balloon-like sacs at the end of the small air passages in the lungs known as bronchioles. In the alveoli, white blood cells called macrophages ingest the inhaled *M. tuberculosis* bacilli ~~(reviewed by van Crevel *et al.*, 2002)~~. Some of the bacilli may be killed immediately and others may multiply within the macrophages. Infrequently, but especially in HIV-infected people and in children, the bacilli spread to other sites in the body (reviewed by van Crevel *et al.*, 2002). This dissemination sometimes results in life-threatening meningitis and other problems.

During the two to eight weeks after initial infection in people with intact immune systems, macrophages present pieces of the bacilli, displayed on their cell surfaces to another type of white blood cell, the T cell. Upon stimulation, T cells release an elaborate array of chemical signals. Once cell-mediated hypersensitivity is established, a person's T cells usually will respond to the tuberculin skin test (PPD test) and produce a characteristic red welt (NIAID, 1997). Some of the T cell signals produce inflammatory reactions, other signals recruit and activate specialized cells to kill bacilli and wall-off the infected macrophages in tiny, hard grayish capsules known as tubercles.

From then on the body's immune system maintains a standoff with the infection, sometimes for years. TB bacilli may persist within macrophages but further multiplication and spreading of *M. tuberculosis* are ~~confined~~restricted. Most people undergo complete healing of their initial infection and the tubercles calcify and lose their viability. A positive TB skin test and in some cases a chest x-ray may provide the only evidence of the infection. If, however, the body's resistance is low because of aging, infections such as HIV, malnutrition or other factors, the bacilli may break out of the tubercles in the alveoli and lead to active disease (Kaufmann & Ulrichs, 2003).

The phagocytosis and the subsequent secretion of IL-12 are processes initiated in the absence of prior exposure to an antigen and hence form a component of innate immunity. The components of innate immunity are natural resistance associated macrophage protein (*Nramp 1*), neutrophils, natural killer cells (NK) and Toll-like receptors (TLR). Plasma lysozyme and other enzymes also may

play an important role in the first line defence of innate immunity to *M. tuberculosis* (Selvaraj *et al.*, 2001).

*Nramp 1* is crucial in transporting nitrite from intracellular compartments such as the cytosol to more acidic environments like phagolysosome, where it can be converted to nitric oxide (NO). Defects in *Nramp 1* production increase susceptibility to mycobacteria. However, *Nramp 1* gene might not be associated with the susceptibility to pulmonary and spinal TB in the Indian population (Selvaraj *et al.*, 2002). Increased accumulation of neutrophil in the granuloma and increased chemotaxis has suggested a role for neutrophils (Edwards *et al.*, 1986). Majeed *et al.* (1998) have shown that neutrophils can bring about killing of *M. tuberculosis* in the presence of calcium under *in vivo* conditions.

Natural killer (NK) cells are also the effector cells of innate immunity. These cells may directly lyse the pathogens or can lyse infected monocytes. NK culture with live *M. tuberculosis* brought about the expansion of NK cells implicating that they may be an important responders to *M. tuberculosis* infection *in vivo* (Esin *et al.*, 1996). During early infection, NK cells are capable of activating phagocytic cells at the site of infection. IL-2 activated NK cells can bring about mycobacterial activity in macrophages infected with *M. avium* complex (MAC) as a non-specific response (Bermudez & Young, 1991). NK cells can also produce IFN- $\gamma$  and can lyse mycobacterium-pulsed target cells (Molloy *et al.*, 1993). Furthermore, augmentation of NK activity with cytokines implicates them as a potential adjunct to TB chemotherapy (Nirmala *et al.*, 2001).



TLR is another component that plays an important role in immunity against TB. The interactions between *M. tuberculosis* and TLRs are complex and it appears that distinct mycobacterial components may interact with different members of the TLR family. *M. tuberculosis* can immunologically activate cells via either TLR2 or TLR4 in a CD14-independent, ligand-specific manner (Means *et al.*, 1999).

### 1.7.2 Host specific immune mechanisms in TB

*M. tuberculosis* is equipped with numerous immune evasion strategies, including modulation of antigen presentation to avoid elimination by T cells. *M. tuberculosis*-infected macrophages appear to be diminished in their ability to present antigens to CD4<sup>+</sup> T cells, which leads to persistent infection (Hmama *et al.*, 1998). Another mechanism by which antigen presenting cells (APCs) contribute to defective T cell proliferation and function is by the production of cytokines, including TGF- $\beta$ , IL-10 (Rojas *et al.*, 1999) or IL-6 (van Heyningen *et al.*, 1997). In addition, it has also been reported that virulent mycobacteria were able to escape from fused phagosomes and multiply (Moreira *et al.*, 1997).

#### 1.7.2(a) Humoral immune response

Since *M. tuberculosis* is an intracellular pathogen, ~~the~~ serum components may not get access to it and may not play any important protective role. However, a Although many researchers have dismissed a role for B cells or antibody in protection against TB (Johnson *et al.*, 1997), recent studies suggest that these immune components may contribute to the response to TB (Bosio *et*

*al.*, 2000). Several protein antigens of *M. tuberculosis* have been identified using murine monoclonal antibodies (Lingers *et al.*, 1986). The immunodominant antigens for mice include 71, 65, 38, 23, 19, 1.4 and 12 kDa proteins.

A number of *M. tuberculosis* antigens have been purified for diagnostic used (Lima Devi *et al.*, 2001, Demkow *et al.*, 2002, Raja *et al.*, 2002 and Ramalingam *et al.*, 2002). Combinations of antigens ~~was~~were ~~also~~ found to be useful in the diagnosis of TB (Ramalingam *et al.*, 2003). Detection of circulating immune complex-bound antibody was found to be more sensitive ~~as compared~~ to serum antibodies. ~~The purified antigens were evaluated for their utility in diagnosing infection (Senthil Kumar *et al.*, 2000).~~

### 1.7.2(b) Cellular immune response

*M. tuberculosis* is a pathogen for which the protective response relies on cell mediated immunity (CMI). In the mouse model, within one week of infection with virulent *M. tuberculosis*, the number of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the lung draining lymph nodes increases (Feng *et al.*, 1999). In principle, three processes that ~~contributes~~contribute to the cellular immune response are; antigen presentation, co-stimulation and cytokine production (van Crevel *et al.*, 2002).

Mycobacterial antigens presentation by macrophages and dendritic cells trigger T-cells response in the context of major histocompatibility complex (MHC). MHC molecules ~~include~~ class I and class II ~~are involved in for the~~ presentation of mycobacterial antigens (Flynn, 2004). Antigen presentation involves CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells and unconventional T-cells including CD1 restricted T cells (van Crevel *et al.*, 2002).

After processing mycobacterial antigens in ~~the~~ phagolysosomal compartments of professional antigen-presenting cells (APCs), MHC class II molecules present these mycobacterial proteins to antigen specific CD4<sup>+</sup> T cells. The effector functions of CD4<sup>+</sup> T cells are sufficient to activate macrophages. Basically, the effector function of CD4<sup>+</sup> T cells can be further divided into two, which are Th1 and Th2 type.

Th1 secretes cytokines such as IL-2, IFN- $\gamma$ , TNF- $\alpha$  and IL-12 resulting in macrophage activation and induction of CMI. Most data obtained from animal

models suggest that Th1 cells and IFN- $\gamma$  are the major effectors that lead to macrophage activation (Kaufmann & Anderson, 1998). In contrast, Th2 secretes IL-4, IL-5, IL-6 and IL-10 resulting in the induction of humoral immunity by antibody production. The HIV epidemic has demonstrated that the loss of CD4<sup>+</sup> T cells greatly increases susceptibility of the host to both acute and reactivation TB (reviewed by Flynn, 2004).

Meanwhile, MHC class I molecules present mycobacterial proteins to antigen-specific CD8<sup>+</sup> T-cells, which will lead to the killing of infected cells from following peptide-MHC recognition. This cytolytic functions of cytotoxic T lymphocytes (CTL) releases a cytolytic molecule, perforin which will induce apoptosis in infected cells (Esser *et al.*, 2003). It was suggested that CTL killing of the bacteria depends on their ability to deliver potent bactericidal proteins such as granulysin from their granules (Silva *et al.*, 2001). Lysise of target cells by CD8<sup>+</sup> T cells can occur via perforin and granzymes or the Fas/FasL (CD95L) pathway resulting in apoptotic cell death or release of bacteria from an infected cell into the granuloma (Canaday *et al.*, 2001).

As mentioned earlier, unconventional T cells such as CD1 and  $\gamma\delta$  T cells also appear to play a have roles in host defense against mycobacterial infection. Both cells produce type-1 cytokines, most importantly IFN- $\gamma$  which activates anti-mycobacterial activities in macrophage (Raupach & Kaufmann, 2001). CD1 restricted  $\alpha\beta$  T-lymphocytes are thought to be activated by mycobacterial

lipids whereas  $\gamma\delta$  T-lymphocytes are activated by small phosphorylated metabolites (Agger & Anderson, 2002).

Both  $CD4^+$  and  $CD8^+$  T lymphocytes as well as unconventional T cells such as  $\gamma\delta$  T-cells and double-negative  $\alpha\beta$  T-cells subsets are involved in immunity to TB (Andersen, 2001). The pulmonary immune response in resistant mice was characterized by an early influx of both  $CD4^+$  and  $CD8^+$  lymphocytes that produced IFN- $\gamma$  (Alissa *et al.*, 2001). These results suggest an important role for the early appearance of IFN- $\gamma$  producing lymphocytes in the lung in resistance to *M. tuberculosis* infection ~~with *M. tuberculosis*~~.

A study on animal models of TB have suggested that both  $CD4^+$  and  $CD8^+$  T-cells have ~~a protective roles~~ in *M. tuberculosis* infection (Cooper and Flynn, 1995). Within one week of infection with virulent *M. tuberculosis*, the number of activated  $CD4^+$  and  $CD8^+$  T-cells in the lung during lymph nodes increases (Serbina *et al.*, 2000). Meanwhile, between 2 to 4 weeks post-infection, both  $CD4^+$  and  $CD8^+$  T-cells migrate to the lungs and demonstrate an effector phenotype (Feng *et al.*, 1999 and Serbina *et al.*, 2000). Both  $CD4^+$  and  $CD8^+$  T-cells are also found in the granuloma to prevent spreading or reactivation (Flynn *et al.*, 1992).

Co-stimulation is the second process that leads to the initiation of cellular immunity. Antigen presentation only leads to T cell stimulation in the presence of several co-stimulatory signals. B-7.1 (CD80) and B-7.2 (CD86) are the most well known co-stimulatory signals for T cell stimulation. These co-stimulatory

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