EVALUATION OF PRIME BOOSTING VACCINATION STRATEGY USING NEWLY CONSTRUCTED TUBERCULOSIS VACCINE CANDIDATES IN MICE

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by

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DEDICATIONS

This thesis is specially dedicated to:

My beloved husband, Nor Affandi Mohamed Adzha

My daughter, Nur Maisarah Binti Nor Affandi

My parents, Zakaria Bin Shaari and Maimun Binti Ismail

My little brother, Muhammad Akmal Hakim Bin Zakaria

Thank you for your endless love, support and encouragement.

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LIST OF ABREVIATIONS

AFB	Acid fast bacillus
AIDS	Acquired Immunodeficiency Syndrome
Amp	Ampicillin
BCE	Before century
BCG	Bacillus Calmette Guerin
bp	Base pair
CFU	Colony forming unit
Con A	Concanavalin A
Co_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
ddH_2O	deionized distilled water
DC	Dendritic cell
DNA	Deoxyribonucleic acid
γδ	Gamma delta
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
MDR-TB	Multi-drug-resistant tuberculosis
MHC	Major histocompatibility complex
NK	Natural killer

OD	Optical density
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPD	Purified protein derivatives
rBCG	Recombinant BCG
RE	Restriction enzyme
RNA	Ribonucleic acid
TB	Tuberculosis
Th	T helper
TNF	Tumour necrosis factor
UV	Ultra violet
WHO	World health Organization
XDR-TB	Extensively drug-resistant tuberculosis

PENILAIAN STRATEGI 'PRIME BOOST' MENGGUNAKAN CALON VAKSIN TUBERKULOSIS YANG BARU TERHADAP MENCIT

ABSTRAK

Tuberkulosis, penyakit berjangkit yang disebabkan oleh *Mycobacterium tuberculosis complex*, merupakan masalah kesihatan yang utama di dunia. Pada masa kini, vaksin yang digunakan untuk mencegah penyakit TB ialah Bacillus Calmette Guerin (BCG), tetapi keberkesanan suntikan BCG masih kontroversi. Pelbagai pendekatan pemberian vaksin telah diolah berdasarkan kepada teknologi masakini. Dalam kajian ini, kami menggunakan dua calon vaksin yang sebelumnya telah dihasilkan, yang dinamakan vaksin DNA, VacIV dan StVacIII 'surface display vaccine' bersama-sama dengan vaksin BCG bagi memanfaatkan strategi vaksinasi 'prime boosting'. Vaksin DNA, VacIV telah diberikan secara intraotot kepada mencit manakala vaksin 'surface display', StVacIII dan BCG telah diberikan secara oral. Darah dan splenosit daripada mencit yang diimunisasi telah diuji dengan pelbagai ujian keimunan. Keputusan menunjukkan bahawa darah ('mice whole blood') dan splenocit daripada mencit yang diimunisasi didapati adanya peningkatan penghasilan IL-2 and IFN-γ apabila dirangsang dengan antigen (Mtb 8.4) yang merupakan salah satu 'epitopes' yang terdapat dalam vaksin DNA, VacIV dan VacIII. Analisis sitokin intrasel ke atas splenosit mencit yang diimunisasi menunjukkan kedua-dua CD4+ dan CD8+ sel T turut menghasilkan IL-2 dan IFN-γ berikutan rangsangan antigen. Tindakbalas yang sama juga dilihat dalam 'peripheral blood'. Dalam kaedah 'prime-boost', kajian menunjukkan mencit yang di 'prime' dengan StVacIII dan 'boost' dengan vaksin DNA, VacIV adalah strategi yang

terbaik untuk meningkatkan respon keimunan mencit. Secara kesimpulannya, data yang diperolehi daripada kajian ini mencadangkan bahawa penggabungan vaksin 'surface display' dan vaksin DNA menggunakan kaedah 'prime-boost' memberi idea baru dalam perkembangan vaksin terhadap tuberkulosis. Kajian lebih lanjut diperlukan untuk memastikan keberkesanan strategi vaksinasi 'prime-boosting' dari aspek perlindungan terhadap model haiwan.

EVALUATION OF PRIME BOOSTING VACCINATION STRATEGY USING NEWLY CONSTRUCTED TUBERCULOSIS VACCINE CANDIDATES IN MICE

ABSTRACT

Tuberculosis (TB) an infectious disease caused by *Mycobacterium tuberculosis complex* continues to be major health problem, worldwide. Current the only available preventive TB vaccine used is *Bacillus Calmette Guerin* (BCG) but unfortunately, the efficacy of BCG nowdays is controversial. Different vaccine delivery approaches have been developed based on the available technologies. In this study, we are using two previously constructed vaccine candidates namely VacIV DNA vaccine and StVacIII surface display vaccine, together with standard BCG vaccine employing prime boosting vaccination strategy. VacIV DNA vaccine was given intramuscularly to mice while StVacIII surface display vaccine and BCG was given orally. Mice whole blood and splenocytes from the vaccinated mice were tested for various immunological tests. The results showed that mice whole blood (peripheral blood) and splenocytes from the immunized mice were found to increase the production of IL-2 and IFN-γ when stimulated with the antigen (Mtb 8.4) which is one of the epitopes in both VacIV and VacIII DNA vaccine. Flow cytometric intracellular cytokine analysis of splenocytes from vaccinated mice showed that both CD4+ and CD8+ T cells produce IL-2 and IFN-γ upon stimulation with the antigens. The same responses also were seen in peripheral blood. In the prime-boost approach, the study showed that mice primed using StVacIII surface display vaccine and boosted with VacIV DNA vaccine is a better strategy in increasing the immune response in mice. In conclusion, the data obtained from this study suggested that

surface display vaccine in combination with DNA vaccine using prime-boost vaccination strategy gives new ideas in vaccine development against tuberculosis. Further study is required to confirm the efficacy of the prime–boosting vaccination strategy in term of protection in animal model.

CHAPTER ONE

INTRODUCTION

1.1 Background of TB

1.1.1 Introduction to TB

Tuberculosis (TB) is one of the most overwhelming infectious diseases accounting for enormous human misery (WHO, 2006). It is caused by *Mycobacterium tuberculosis complex* (*M. tuberculosis complex*). It is estimated that three billion people are infected with around two million deaths attributable to tuberculosis (TB) per annum (Frieden *et al.*, 2003). According to Zumla *et al.*, (2000), it is predicted that in the period of 2000-2020, another billion of people will be infected with *M. tuberculosis* in which 200 million will develop TB and 35 million will die from the disease. This phenomenon is crucially will impact the socio-economic on a community as this kind of disease also affects individuals between 20-45 age groups during their peak period of productivity.

1.1.2 History of TB

M. tuberculosis is a bacterium that causes TB in human population since long time ago. One of the evidence is where fragments of the spinal column from Egyptian mummies from 2400 BCE had been found. This artificial finding show definite signs of tuberculosis (Zink *et al.*, 2003).

Around 460 BCE, Hippocrates has found 'Phthisis'; the term refers to consumption (one of the first terms used during Greek literature) is the most widespread disease. Furthermore, he noted that it was almost always fatal. He warned his colleagues not to visit TB patients who were in the late stages of the disease as their inevitable deaths might damage the reputations of the attending physicians (Madkour *et al.*, 2004).

On 24 March 1882, *M. tuberculosis* was identified by Robert Koch using a staining technique that enabled him to see *M. tuberculosis.* He received the Nobel Prize (physiology or medicine) in 1905 for this discovery. Koch did not believe that bovine (cattle) and human tuberculosis were similar. This caused a delay in the recognition of infected milk as source of infection. In, the process the source was shown to be eliminated by pasteurization. Lastly, in 1890, Koch announced a glycerine extract of the tubercle bacilli as a treatment for tuberculosis which he called 'tuberculin'. Due to the ineffectiveness of treatment of the disease, 'tuberculin' was later adapted on a test for pre-symptomatic tuberculosis (Waddington, 2004).

Another important discovery was provided by the French bacteriologist known as Calmette who works together with Guerin, used the specific culture media to lower the virulence of the *M. bovis*, resulting in the advent of Bacillus Calmette Guerin (BCG) vaccine (Bonah, 2005; Comstock, 1994).

This first successfully BCG vaccine was used on human in 1921 in France (Bonah, 2005) but it was not until after World War II that BCG received widespread acceptance in USA, Great Britain and Germany (Comstock, 1994).

1.1.3 Epidemiology of TB

1.1.3(a) Prevalence of TB in the world

The number of TB notifications had increased for over the last 10 years. Based on the latest data of core health indicators from World Health Organization (WHO) 2006, the prevalence of TB per 100, 000 populations had increased to the value of 130.9 in 2005. Furthermore, incidence of TB per 100, 000 populations per year is about 101.6.

TB is a leading infection causing deaths among people more than 5 years of age in south-East Asia and accounts for approximately 34 percent of TB cases in the world and it shows the most cases compared to another WHO region (Table 1.1) (Figure 1.1a and 1.1b).

Table 1.1: Estimated TB Incidence, Prevalence and Mortality, 2005. (Adapted from WHO,2006).

	Incidence[a]				Prevalence[a]		TB Mortality	
	All forms		Smear-positive[b]					
WHO region	number (thousands) (% of global total)	Per 100 000 pop	number (thousands)	per 100 000 pop	number (thousands)	Per 100 000 pop	number (thousands)	Per 100 000 pop
Africa	2 529 (29)	343	1 088	147	3 773	511	544	74
The Americas	352 (4)	39	157	18	448	50	49	5.5
Eastern Mediterranean	565 (6)	104	253	47	881	163	112	21
Europe	445 (5)	50	199	23	525	60	66	7.4
South-East Asia	2 993 (34)	181	1 339	81	4 809	290	512	31
Western Pacific	1 927 (22)	110	866	49	3 616	206	295	17
Global	**8 811 (100)**	**136**	**3 902**	**60**	**14 052**	**217**	**1 577**	**24**

[a]Incidence - new cases arising in given period; prevalence - the number of cases which exist in the population at a given point in time.
[b]Smear-positive cases are those confirmed by smear microscopy, and are the most infectious cases.
pop indicates population.

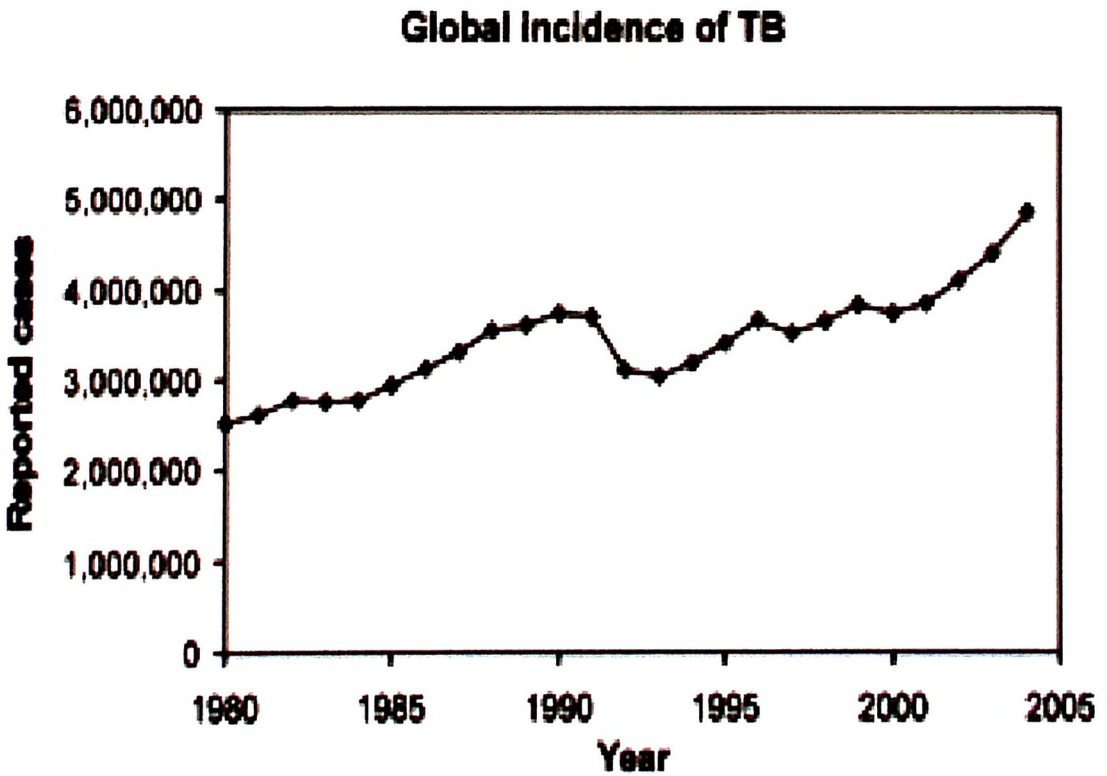

Figure 1.1a : Annual number of new reported TB cases (Adapted from WHO, 2006)

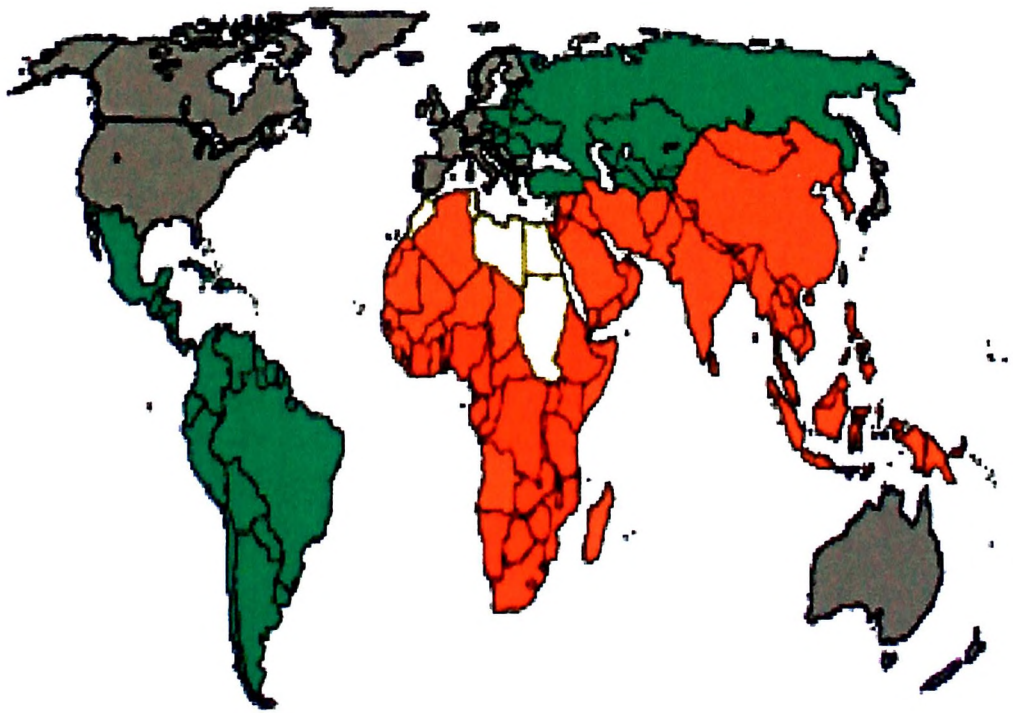

Figure 1.1b :World TB incidence. Cases per 100,000; Red = > 300, orange = 200-300; yellow = 100- 200; green 50 -100 and grey < 50. (Data from WHO, 2006)

1.1.3(b) Prevalence of TB in Malaysia

Statistics from the Ministry of Health Malaysia (MOHM) in 2006, stated that Sabah led the other states in Malaysia with 3319 cases of TB infection in 2005 (Jiloris F. *et al.*, 2004) , followed by Selangor with an increasing number of cases from 1,874 in 2004 to 1986 cases in 2005, with Sarawak 1502 cases, and Kelantan, 1150 cases. The number of deaths had increased each year (Jiloris *et al.*, 2004; Nik Nor Rohaidi *et al.*, 2011).

The latest data from MOHM indicated that the number of TB cases in the country had increased annually and about 17, 000 cases were reported in 2010. 1005 patients died from the disease. One of the contributing factors listed of the increasing number of TB cases was the co-infection between TB and Human immunodeficiency virus (HIV) (WHO Regional Committee for the Western Pacific Region meeting, 2010).

1.1.4 Bacteriological aspects of *M. tuberculosis*

The main cause of TB, which is *Mycobacterium tuberculosis* (*M. tuberculosis*) (Figure 1.2) is an aerobic bacterium that segregate every 16 to 20 hours which show an extremely slow growth rate compared with other bacteria, which usually segregate in less than an hour (Cox, 2004), with the fastest-growing bacteria being strain of *Escherichia coli* which can divide roughly every 20 minutes. Stated by Sohaskey *et al.*, (2003) this *M. tuberculosis* undergoes a metabolic downshift in oxygen limit in environment of granuloma and switch to anaerobic nitrate respiration. This condition helps in the persistence of the bacilli in anaerobic condition respiration *M. tuberculosis* has a cell wall but lack of the phospholipids outer membrane, so it is classified as a Gram-positive bacterium. *M. tuberculosis* is a small rod-like bacillus that can resist disinfectants and survive in a dry state for weeks. In nature, this bacterium can grow only within the cells of a host organism, but *M. tuberculosis* can be cultured in vitro (Parish and Stoker N, 1999).

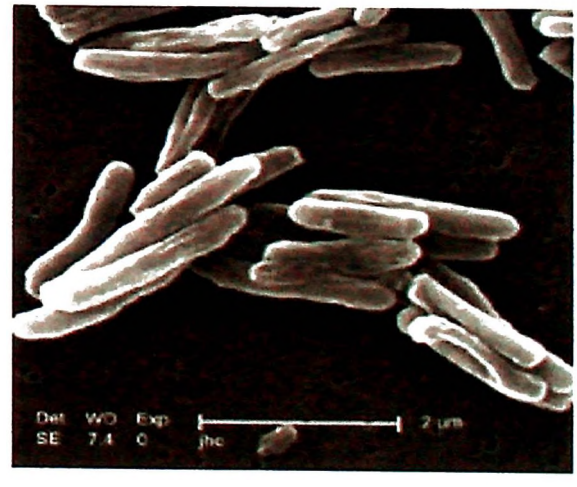

Figure 1.2: Scanning electron micrograph of *Mycobacterium tuberculosis* (Source: http://en.wikipedia.org/wiki/Tuberculosis)

Intracellular pathogens must have strategies to overcome to the poor environment inside the host which is much different in a culture broth outside the host. Bacterial pathogen like *M. tuberculosis* is expected to reallocation of metabolism and synthesize defense molecules to survive the assaults inflicted by alveolar macrophages in the lungs, oxygen and nitric oxide (Srivastava *et al.*, 2007).

M. tuberculosis lives and replicates inside mononuclear phagocytes and immunity crucially depend on T lymphocytes (Ravn *et al.*, 1999).

1.1.5 Tuberculosis in adult and children

Tuberculosis is a major killer of children in poor countries. TB kills more young people than any other single infectious disease. Every minute two children die of TB worldwide. Ten of thousands 'immunised' children in the developing world still suffer from tuberculosis meningitis and other diseases (WHO, 2006).

Tuberculosis infection in children is called primary complex or childhood tuberculosis infection. Children below two years are mostly at risk because they have an under-developed immune system. However, this disease is completely curable and an early diagnosis helps in effective treatment (WHO, 1999).

Being a communicable disease, tuberculosis spreads through the nasal and oral discharge of an infected person. When an infected person coughs or sneezes, the bacteria are released into the air. If an adult have TB that is untreated, he or she can pass the infection to his or her baby as well. Adults who do not complete their TB

treatment might put young children below ten years of age at risk (Flynn and Chan 2001).

Children are highly risk to get tuberculosis. In the first 5 years of life, the power to resist TB infection is normally poor. The resistance can be reduced by malnutrition, HIV, other childhood infections and worm infestations. These examples are – all too common childhood conditions in poor countries (WHO, 2006).

It has been estimated that as many as one third of the world's population is infected with TB, and an estimated 20-50% of children who live in households where an adult has active tuberculosis become infected. Children are especially exposed to infection from household contacts as they are often held close and breathed on. Besides, the risk is quite high in the developing world where family size is large, living quarters are crowded and more than half the population are children (Chintu, 2006).

Another reasons relating on why children have a high risk of developing active TB disease is the immune system of young children is less developed instead of adult. The risk of developing active TB disease is higher in young children. Next, in HIV infected children the risk to develop TB meningitis is very high. Another reason is the relation between tuberculosis and malnutrition which often go together. A child with TB disease may present as failure to gain weight with loss of energy and cough lasting for more than three weeks (WHO, 2006).

As an adult, if the child not looking healthy, has a persistent cough, has repeated chest infections and fever, complains of being tired and not gain enough weight, the doctor may ask for tests to detect the presence of TB causing by bacteria. Additional symptoms include swollen glands and the child may have difficulty breathing.

TB also infected women as well. Perhaps TB is the single biggest killer of young women. According to the World Health Organisation (WHO) it is estimated that TB accounts for 9 percent of deaths which is among women between the ages of 15 to 44. Women of reproductive age are more susceptible to develop active TB once they infected with TB compared to a men of the same age (WHO, 2006).

For example in India, total deaths which caused by TB are 27 to 41% higher among women and children between 5 to 24 years compared to males in the same age (Reported in research by Gender and Health group, Liverpool STM, LATH, Liverpool VCT, Reach Trust, Lilongwe).

After all, TB cases detection seem much lower in women than man (Borgdorff *et al.*, 2000). It is mainly because women delay seeking care so as not to use precious family resources, women are missed by health promotion programs as they tend to stay at home rather than come to the workshops and therefore have lower awareness of TB symptoms, often scared to tell family that they might have TB due to potential rejection, women in some families cannot leave the home without telling where they are going even want to go to a TB clinic and they used to wait up to twice as long to seek for treatment as they are waiting until having severely ill and more likely to die.

1.1.6 Immunopathogenesis of tuberculosis

The chronology of TB infection starts with inhalation of *M. tuberculosis*. When a person inhales the air which contains particles expelled by an infectious person, most of the larger particles become lodged in the upper respiratory tract where infections in unlikely to develop. However, the droplet nuclei may reach the alveoli. This is where the infection normally begins (East African Community Health, 2012) (Figure 1.3).

Initially, the tubercle bacilli will multiply in the alveolar macrophages. A small number of it will spread through the lymphatic channels to regional lymph nodes and next through the bloodstream to more distant tissues and organs, including the place that the disease is most likely to develop, which are the apices of the lungs, the kidneys, the brain and bone (East African Community Health, 2012).

Within 2 to 10 weeks after infection, the immune system usually will intervene, halting multiplication of the tubercle bacilli and preventing further spreading of the disease.

A person who is infected with *M. tuberculosis* but found not to have disease is not infectious to others. An infection in a person who does not have disease is often referred as latent infection. The progression to disease occurs when the tubercle bacilli overcome the defenses of the immune system and begin to multiply.

The progression to disease can occur very quickly or many years after infection. For example TB cases in United State showed in approximately 10 percent of persons infected with *M. tuberculosis* will developed the disease about the same point in time while the remaining 90 percent will stay infected but free from the disease for the rest of their lives.

Meanwhile, some medical conditions might increase the risk of developing the disease. The risk is about 3 times greater (among patients with diabetis mellitus) to 100 times (for those affected by HIV) (Flynn and Chan 2001).

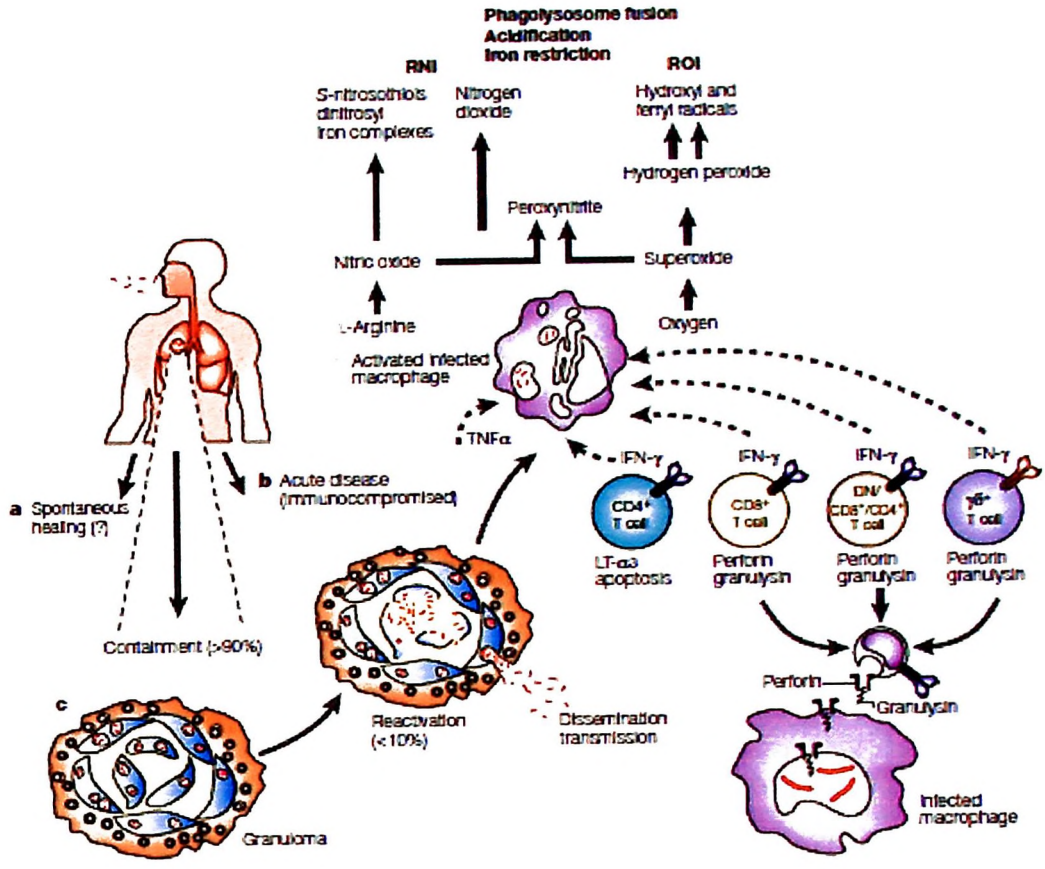

Figure 1.3: Main features of tuberculosis: from infection to host defence. There are three potential outcomes of infection of the human host in Mycobacterium tuberculosis. **a)** The frequency of abortive infection resulting in spontaneous healing isunknown, but is assumed to be minute. **b)** In the immunocompromised host, disease can develop directly after infection.**c)** In most cases, mycobacteria are initially contained and disease develops later as a result of reactivation. The granuloma is the site of infection, persistence, pathology and protection. Effector T cells (including conventional CD4+ and CD8+ T cells, and unconventional T cells, such as γδ T cells, and double-negative or CD4/CD8 single-positive T cells that recognize antigen in the context of CD1) and macrophages participate in the control of tuberculosis. Interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α), produced by T cells, are important macrophage activators. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). LT-α3, lymphotoxin-α3 (Adapted from Kaufmann, 2001).

1.1.7 Diagnosis of Tuberculosis

The clinical diagnosis of pulmonary tuberculosis depends mainly on features of prolonged cough, weight loss, with a history of close contact with an infectious TB patient. Several ways can be used in order to identify and diagnose *M. tuberculosis* infection either using the bacteriological or immunological method or both (Kanai, 1990).

Sputum is the most common specimen received for TB diagnosis. The minimum number of bacilli needed to detect its presence in acid-fast stained smears has been estimated to be 5000 to 10000 per ml of sputum. The sensitivity of AFB smear staining has been estimated to vary from 50 percent to over 80 percent. There are two ways of staining which are Ziehl-Neelsen and Kinyoun staining method. Ziehl-Neelsen is a hot acid-fast stain because the slides have to be heated during incubation with fuchsin. In contrast, Kinyoun staining method is a cold acid-fast staining procedure and does not require heating. The Ziehl-Neelsen method is more widely used as it is less expensive and the detection process is easier and quicker (Cambiaso *et al.*, 1990; Kanai, 1990). It is also the recommended technique by the International Union against TB and Lung Disease (IUATLD) and WHO.

Difficulty in diagnosing TB and other mycobacterial infections using sputum is that this test lack of sensitivity and specificity (Tuberculosis Prevention Trial, Madras 1980). Fluorochrome staining methods been used as it is much more sensitive compared to Ziehl-Neelsen. The smears from the fluorochrome staining are examined with a fluorescence microscope using the appropriate power objective,

making this method suitable to use in central or large laboratory but less effective in small laboratories because of the associated cost, equipmet maintenance, and lower specificity (Toman, 1979 reviewed by Unchalee T. and Booncherd K., 2002)

Another method used is one of the newer BACTEC systems like BACTEC TB-460, BACTEC MGIT-960, VersaTrek or BacT/alert 3 D. This system uses radiolabeled CO_2. In the BACTEC TB-460 system; for example once the paired needles have perforated the rubber septum of the vial, the gaseous phase is aspirated and is replaced by air containing 5 percent CO_2. The aspirated gases are then analyzed by a β-counter to quantify the eventual presence of radilabeled CO_2. The rationale of this instrumentation is when viable mycobacteria are present in the culture vial, the radiolabeled palmitic acid is metabolized and radioactive CO_2 is liberated into gaseous phase.

The tuberculin skin test or purified protein derivative (PPD) uses an extract of killed TB bacteria (Frieden *et al.*, 2003). The killed bacteria are injected into the skin. If a person once had been infected with TB, an induration will form at the site of infection. This shows a positive test in PPD. This means that TB germs have infected the body. People with positive skin tests but without active disease cannot spread the infection to others.

If a person has been infected with TB but not an active TB, the chest X-ray often be normal. Most people with a positive PPD have normal chest X-rays and continue the healthy life. But the preventive medication may be recommended. However, if the

bacteria has attacked and caused inflammation in the lungs, the abnormal shadow will appeared on the chest X-rays (Frieden *et al.*, 2003).

Diagnosis of tuberculosis involving children can be difficult especially children under the age of 10 years who usually cannot cough up sputum to be sent for laboratory investigations to confirm ones got TB infection. Special investigations like skin test and a chest X-ray can be helpful in making the diagnosis of TB in children (Osborne, 1995 reviewed by Chintu, 2007).

Until now, a polymerase chain reaction (PCR) assay was used for the detection of *M. tuberculosis*. The first proposed genomic targets for diagnostic PCR was the insertion element of IS6110 which being present in multiple copies from 4 to 20 in more than 95 percent of *M. tuberculosis* strains which appeared to have the potential for enhanced sensitivity (Parvez *et al.*, 2003). Another DNA gene which had been successfully used including the 65 kiloDalton (kDa) heat-shock protein gene, gene encoding 126 kDa fusion protein and gene encoding β-subunit of ribonucleic acid (RNA) polymerase. All of them present in single copies in *M. tuberculosis* complex genomes.

1.1.8 Control of TB

Tuberculosis may affect the central nervous system (meninges, brain or spinal cord) in which the case is called TB meningitis, TB cerebritis and TB myelitis. CNS TB may be secondary to blood-borne spread. Therefore, some experts advocate the routine sampling of CSF in patients with miliary TB (Chang *et al.*, 1998).

The anti-TB drug that are useful for the treatment of CNS TB are INH (CSF penetration 100%), RMP (10%-20%), EMB (25%- 50 % inflamed meninges only), pyrazinamide (PZA) (100%), STM (20% inflamed meninges only), LZD (20%), Cycloserine (80 – 100%), Ethionamide (100%) and p-aminosalicylic acid (PAS) (10% - 50%) which inflamed meninges only. The modern short course chemotherapy involved 2 months PZA, 6 months INH and rifampin (RIF). According to Petrini and Hoffner (1999) INH is important especially to inhibit the synthesis of mycolic acid. Meanwhile, PZA play important roles in the intensive phase of the bacteria (Mitchison, 2005). RIF has bactericidal properties against both intracellular or extracellular *M. tuberculosis* and being important agent in the treatment of TB (Friedman, 2000).

Extensively drug-resistant tuberculosis (XDR-TB) is defined as the *Mycobacterium tuberculosis* which is resistance to rifampicin and isonazid as well as any member of the quinolone family and at least one of the following second-line TB treatments-kanamycin, capreomycin or amikacin (WHO, 2006). The old definition of XDR-TB

is Multi-drug-resistant tuberculosis (MDR-TB) which is also resistant to three or more of the six classes of the second line drugs (Center of Disease Control, 2006).

The principles of both MDR- TB and XDR-TB are the same. The main difference is that XDR-TB is related with a higher mortality compared with MDR-TB because of a reduced number of effective treatment options (Center of Disease Control, 2006). It is believed that XDR-TB does not transmitted easily in a healthy population and yet is capable of causing epidemics in the populations which are already stricken by HIV who are more susceptible to TB infection (Sarah, 2006).

DOTS which stands for "Directly Observed Therapy, Short-course" programme is a major plan by WHO in the global eradication programme of TB. WHO advises that all TB patients should have at least the first two months of their therapy observed where an independent observer will be watching TB patients swallow their anti-TB therapy. The independent observer is often not a healthcare worker who maybe is a shopkeeper or similar senior person within that society. Intermittent dosing (thrice weekly or $2HREZ/4HR_3$) will be used in DOTS therapy. Dosing twice weekly is effective but it is not recommended by WHO as there is no margin for error (omitting one dose per week results in one weekly dosing, which is ineffective). Treatment with properly implemented DOTS has a success rate exceeding 95 percent and in the same time prevents the emergence of further multi-drug resistant strains of tuberculosis (WHO, 2006).

1.2 Human Immune Responses against TB

1.2.1 Human Immune System

The immune system plays very important role in protecting our body from pathogens. It consists of innate and adaptive immune system. In innate immunity, if a pathogen breaches these barriers, it will provide an intermediate, but non-specific response. But if a pathogen is successful in evading the innate response, vertebrates possess a third layer of protection which is the adaptive immune system that maybe activated by the innate response. The immune system adapts its response during an infection to improve the recognition of and response to the pathogen. This improved response is then preserved after the pathogen has been eliminated, in the form of an immunological memory and allows the adaptive immune system faster and stronger counter-attacks each time this pathogen is encountered (Mayer, 2006).

The innate response is usually triggered when microbes are identified by pattern recognition receptors which recognize components that are conserved among various group of microorganisms (Medzhitov, 2007). Innate immune defenses are non-specific which means that these systems respond to pathogens in a various way (Albert *et al.*, 2002). The innate immune system is the dominant system of host defense in most organisms (Litman, 2005).

Innate response includes the response of various leukocytes, in particular cells of mononuclear phagocyte system (macrophage and dendritic cell), granulocytes and natural killer (NK) cells. Leukocytes (white blood cells) are independent, single-celled organisms and being the second arm of the innate immune system.

Cell-mediated immunity is an immune response that does not involve antibodies or other complement system but involves in the activation of macrophages, natural killer cells (NK), antigen specific cytotoxic T-lymphocytes and the release of variable cytokines in response to an antigen.

Cells of the immune response consist of leukocyte, mast cells, phagocytes, macrophages, neutrophils, dendritic cells, basophils and eosinophils, natural killer cells, also γδ T cells.

'Phagocyte' refers to eating cell. These are immune cells that extends engulf portions of its plasma membrane, wrapping the membrane around the particle until it is enveloped. Once the particles are inside the cell, the invading pathogen contained inside an endosome which merges with a lysosome(Janeway *et al.*, 2001). This lysosome contains enzymes and acids which can kill and digest the particle or organism. Phagocytic cells play roles in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and expression of co-stimulatory signals and cytokines. Due to TB infection, according to Bermudez& Goodman, 1996 and Van Crevel *et a*l., 2002) when *M. tuberculosis* first enters the alveolar macrophage, phagocytosis of *M. tb* become the first action taken as a response against this infectious agent as the cells that involved are dendritic cells, alveolar type II pneumocytes and monocyte derived macrophage.

Meanwhile, the adaptive immunity can be divided into 2 main responses which are humoral and cellular immune response. The humoral immune response (HIR) is the

aspect of immunity which is mediated by secreted antibodies (as opposed to cell-mediated immunity which involves T lymphocytes). It is produced in the cells of the B lymphocyte lineage (B cell). Secreted antibodies will bind to antigens on the surfaces of invading microbes such as viruses or bacteria, which flags them for destruction (Pier *et al.*, 2004). Humoral immunity is called as humoral, because it involves substances found in the humours, or body fluids.

Study of the molecular and cellular components being the central science of immunology as it comprises the immune system, including their function and interaction. The immune system is divided into a more primitive innate immune system, and acquired or adaptive immune system of vertebrates, the latter of which is further divided into humoral and cellular components.

Humoral immunity refers to antibody production, and the accessory processes including; Th2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation. It also refers to the effector functions of antibody, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination (Stephan and Keertan, 2011 ; Xueqiong *et al.*, 2010).

Cell-mediated immune response is an important response to control the infection stage in our body especially in *M. tuberculosis* infection. This response provides a major protective immune response to *M. tuberculosis* instead of the humoral responses (Kaufmann, 1995).

Cellular immunity protects the body by activating antigen-specific cytotoxic T-lymphocytes which are able to induce apoptosis in body cells displaying epitopes of the foreign antigen on their surface for example virus-infected cells, cells with intracellular bacteria and cancer cells that display tumor antigens. Furthermore, it activate the macrophage and natural killer cells which enable them to destroy intracellular pathogens and at the same time stimulate cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive and innate immune response (Kaufmann, 1995).

1.2.2 Protective Immunity against TB

The lung is the main entry for *M. tuberculosis* in most of the TB infections which provides a suitable environment for this pathogen. The infection is established in alveolar macrophages of the distal alveoli before it is recognized by the adaptive immune response in five to six weeks later. CD4+ and CD8+ T cells are recruited through the lung to induce the protective immunity (Young *et al.*, 2002).

Basically, both CD4 and CD8 T cells are important for protective immunity against *M. tuberculosis*. Resistance to *M. tuberculosis* involves the activation of mycobacterial-specific CD4+ and CD8+ T cells by dendritic cells (DC), which migrate from the site of the infection in the alveoli to the draining lymph nodes. The development of interferon (IFN)-c-secreting CD4 T cells is dependent on the secretion of interleukin (IL)-12 by the infected DC. Flynn and Chan (2001) stated that subjects deficient in receptors for IFN-c and IL-12 are extremely susceptible to

mycobacterial infections, confirming the absolute requirement for T-helper cell type 1 (Th1)-like T cells for host immunity.

The effective immune response against TB is incompletely understood but the most effective vaccination strategies in animal models are those that can stimulate T-cell responses, both CD4 and CD8, to produce Th1-associated cytokines. Furthermore, formulations that induce the production of permanent Th1 responses are desirable, and doubtless an essential element of a successful vaccine.

1.3 TB Vaccine Development

1.3.1 Failure in BCG Vaccination

Bacillus Calmette-Guérin (BCG), an attenuated strain of M. *bovis* has been widely used for vaccination against human tuberculosis (Fine, 1995). BCG vaccination was first introduced in 1921. Immunization with BCG in infancy provides protection against childhood forms of disseminated TB and leprosy (Trunz, 2006) but after all it is ineffective in protecting against adult pulmonary disease, especially in TB endemic (Fine, 1995) .

The World Health Organization (WHO) recommends this type of vaccination in areas of high TB prevalence and incidence. BCG vaccination is currently compulsory in more than 64 countries and administered in more than 167 countries (WHO, 1995).