# DEVELOPMENT OF RECOMBINANT *Mycobacterium smegmatis* (MS) EXPRESSING B- AND T-CELL EPITOPES OF LATENCY ASSOCIATED ANTIGENS OF *Mycobacterium tuberculosis* (MTB) AS A TB VACCINE CANDIDATE

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**by**

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## LIST OF SYMBOLS

| Symbol | Definition |
|---|---|
| % | Percentage |
| > | More than |
| < | Less than |
| $^{o}C$ | Degree Celsius |
| β | Beta |
| ~ | Approximately |
| ™ | Trade mark |
| α | Alpha |
| γ | Gamma |
| δ | Delta |
| μ | Micro |
| λ | Lambda |

## LIST OF ABBREVIATIONS

| Abbreviation | Definition |
|---|---|
| Abs | Absorbance |
| Ag | Antigen |
| APC | Antigen Presenting Cell |
| bp | base pair |
| BCG | Bacille Calmette Guerin |
| CD | Cluster of Differentiation |
| CDC | Centre for Disease Control and Prevention |
| CFU | Colony Forming Unit |
| CFSE | CellTrace™ CFSE Cell Proliferation Kit |
| cm | Centimeter |
| CMI | Cell Mediated Immunity |
| CTL | Cytotoxic T Lymphocyte |
| dd$H_20$ | deionised distilled water |
| DNA | Deoxyribonucleic Acid |
| DosR | Dormancy Regulator |
| DOTS | Directly Observed Therapy/Treatment Short course |
| ECL | Enhanced Luminol-based Chemiluminescent |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| *et al.* | and others |
| EtBr | Ethidium Bromide |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| EPTB | Extra-pulmonary TB |
| ETH | Ethambutol |
| FCS | Fetal Calf Serum |
| FITC | Fluorescein Isothiocynate |
| FM | Foamy Macrophage |
| HIV | Human Immunodeficiency Virus |
| hr | Hour |
| HRP | Horseradish Peroxidase |
| Hsp | Heat shock proteins |
| IFN-γ | Interferon-gamma |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IGRA | Interferon-Gamma Release Assay |
| INH | Isoniazid |
| kDa | kilo Dalton |
| kV | Kilovolt |
| LAM | Lipoarabinomannan |
| LTBI | Latent TB Infection |
| M | Molar |
| mA | milliAmpere |
| mAb | monoclonal Antibody |
| MDG | Millennium Development Goal |
| MDR | Multi Drug Resistant |

| | |
|---|---|
| MHC | Major Histocompatibility Complex |
| min | Minute |
| ml | Milliliter |
| mm | Millimeter |
| MOH | Ministry of Health |
| MS | *Mycobacterium smegmatis* |
| MTB | *Mycobacterium tuberculosis* |
| n | sample size |
| NAAT | Nucleic Acid Amplification Technique |
| ng | Nanogram |
| nm | Nanometer |
| NK | Natural Killer |
| OADC | Oleic Acid/ Albumin/ Dextrose/ Catalase enrichment |
| OD | Optical Density |
| ORF | Open Reading Frame |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PAS | Para Aminosalicylic Acid |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction |
| PE | Phycoerythrin |
| PerCP | Peridinin Chlorophyll Protein |
| PPD | Purified Protein Derivative |
| PZA | Pyrazinamide |
| rBCG | recombinant BCG |
| RD | Region of Difference |
| RIF | Rifampicin |
| rMS | recombinant MS |
| ROI | Reactive Oxygen Intermediate |
| rpm | revolution per minute |
| Rpfs | Resuscitation promoting factors |
| RT | Room Temperature |
| RUTI | Therapeutic vaccine made of detoxified, fragmented MTB cells |
| SD | Standard Deviation |
| RV | Reverse Vaccinology |
| Rv | Region of variance |
| SDA | Strand Displacement Amplification |
| SDS | Sodium Dodecyl Sulphate |
| sec | Second |
| SM | Streptomycin |
| TB | Tuberculosis |
| TLR | Toll-Like Receptor |
| TBM | Tuberculous Meningitis |
| TNF | Tumor Necrosis Factor |
| Th | T helper |
| TST | Tuberculin Skin Test |
| U | Unit |
| UNITAID | International facility for the purchase of diagnostics and medicines for diagnosis and treatment of HIV/AIDS, Malaria and TB |
| UV | Ultraviolet |
| WHO | World Health Organization |

**PEMBANGUNAN REKOMBINAN *Mycobacterium smegmatis* (MS) YANG MENGEKSPRES EPITOP-EPITOP SEL B DAN SEL T DARIPADA ANTIGEN BERKAITAN LATENSI *Mycobacterium tuberculosis* (MTB) SEBAGAI CALON VAKSIN TB**

## ABSTRAK

Tuberkulosis (TB) masih lagi menjadi masalah kesihatan yang utama di dunia. Hampir satu pertiga daripada penduduk dunia dijangkiti *Mycobacterium tuberculosis* (MTB) secara laten dan 5 - 10 % daripada individu yang dijangkiti akan mendapat penyakit TB aktif. Bacille Calmette–Guérin (BCG) sangat berkesan dalam melindungi penyakit TB pada kanak-kanak, tetapi gagal untuk menghalang TB laten atau pengaktifan semula penyakit TB pada orang dewasa. Oleh itu, pembangunan vaksin TB yang berkesan menjadi keutamaan dalam usaha untuk mengawal penyakit ini daripada bertambah buruk. Pengekspresan antigen MTB dalam *Mycobacterium smegmatis* (MS) adalah salah satu strategi yang berpotensi untuk pembangunan vaksin generasi baru terhadap TB. Tesis ini mengkaji proses penghasilan dan menilai tahap immunogenisiti rekombinan MS (rMS) dalam mencit Balb/C yang mengandungi epitop sel B dan sel T daripada Antigen Berkaitan Latensi (LAA) MTB (Rv2005c, Rv2031c, Rv3130c, Rv3127) (rMS081). Kapasiti rMS081 untuk mendorong tindak balas imun humoral dan selular khusus terhadap epitop sel B dan sel T telah dikaji. Jumlah Imunoglobulin G (IgG) spesifik menunjukkan peningkatan signifikan terhadap semua epitop LAA dalam serum mencit yang diimunisasi dengan rMS081 berbanding kumpulan mencit kawalan. Tiada peningkatan ketara dalam penghasilan sitokin Interferon-gamma (IFN-γ), Interleukin (IL)-2 dan IL-4 terhadap epitop LAA dalam mencit yang diimunisasi dengan rMS081 berbanding kumpulan kawalan. Hasil daripada kajian ini dapat menyokong penilaian masa depan rMS081 sebagai calon vaksin terhadap TB.

**DEVELOPMENT OF RECOMBINANT *Mycobacterium smegmatis* (MS) EXPRESSING B- AND T-CELL EPITOPES OF LATENCY ASSOCIATED ANTIGENS OF *Mycobacterium tuberculosis* (MTB) AS A TB VACCINE CANDIDATE**


## ABSTRACT

Tuberculosis (TB) remains a major public health problem worldwide. Nearly one-third of the world population is latently infected with *Mycobacterium tuberculosis* (MTB) and 5 - 10 % of infected individuals will develop active disease during their life time. Bacille Calmette–Guérin (BCG) efficiently protects against severe disease manifestations in children, but does not prevent the establishment of latent TB or reactivation of TB pulmonary disease in adult life. Therefore, in order to control this scourge from exacerbating, the development of an effective TB vaccine is an urgent priority. The expression of MTB antigens in *Mycobacterium smegmatis* (MS) is one of the potential strategies for the development of new generation vaccines against TB. This study focused on the construction and evaluation of immunogenicity in Balb/C mice of a recombinant MS (rMS) expressing B- and T-cell epitopes of Latency-Associated Antigens (LAA) (Rv2005c, Rv2031c, Rv3130c, Rv3127) of MTB (rMS081). The capacity of rMS081 to induce specific humoral and cellular immune responses against expressed B- and T-cell epitopes was evaluated. Total specific Immunoglobulin G (IgG) showed a significant increase against all epitopes (Rv2005c, Rv2031c, Rv3130c, Rv3127) in the sera of rMS081-immunized mice compared with the control group. In the experimental conditions evaluated, there were no significant increase in the cytokines, Interferon-gamma (IFN-γ), Interleukin (IL)-2 and IL-4 against the LAA epitopes in the rMS081-immunized mice compared with the control group. The results obtained support the future evaluation of rMS081 as a vaccine candidate against TB in challenge studies.

# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Tuberculosis (TB)

TB is still a major infectious disease threat to humans worldwide. Globally, approximately 9.6 million of new TB cases were recorded and 1.5 million of TB deaths occurred in 2014 (WHO, 2015). *Mycobacterium tuberculosis* (MTB) can persist within the human host for years without showing disease symptoms, a condition known as latent TB. It caused latent infection in more than one-third of the world population (WHO, 2015). The high prevalence of latent TB infection (LTBI) is one of the main factors that contribute to the increasing incidence of active TB. Thus, research on how MTB establishes a latent metabolic state and the development of new methods to eliminate LTBI are priorities for the future control of TB.

## 1.2 Global incidence of TB

WHO reported that the decreasing rate per year was 1.5 % between 2000 and 2013 and started to fall, 2.1 % between 2013 and 2014, achieving the Millennium Development Goals (MDG) target ahead of 2015 (WHO, 2015). According to the WHO TB report 2014, most of the cases occurred in Asia (58 %) and Africa (28 %), followed by Eastern Mediterranean (8 %), Europe (3 %) as well as other Regions in the Americas (3 %). In 2014, WHO also reported six countries that showed the highest TB cases were India (2.2 million), Indonesia (1.0 million), China (0.93 million), Nigeria (0.57 million), Pakistan (0.5 million) and South Africa (0.45 miilion). Figure 1.1 shows the estimated number of new TB cases in 2014.

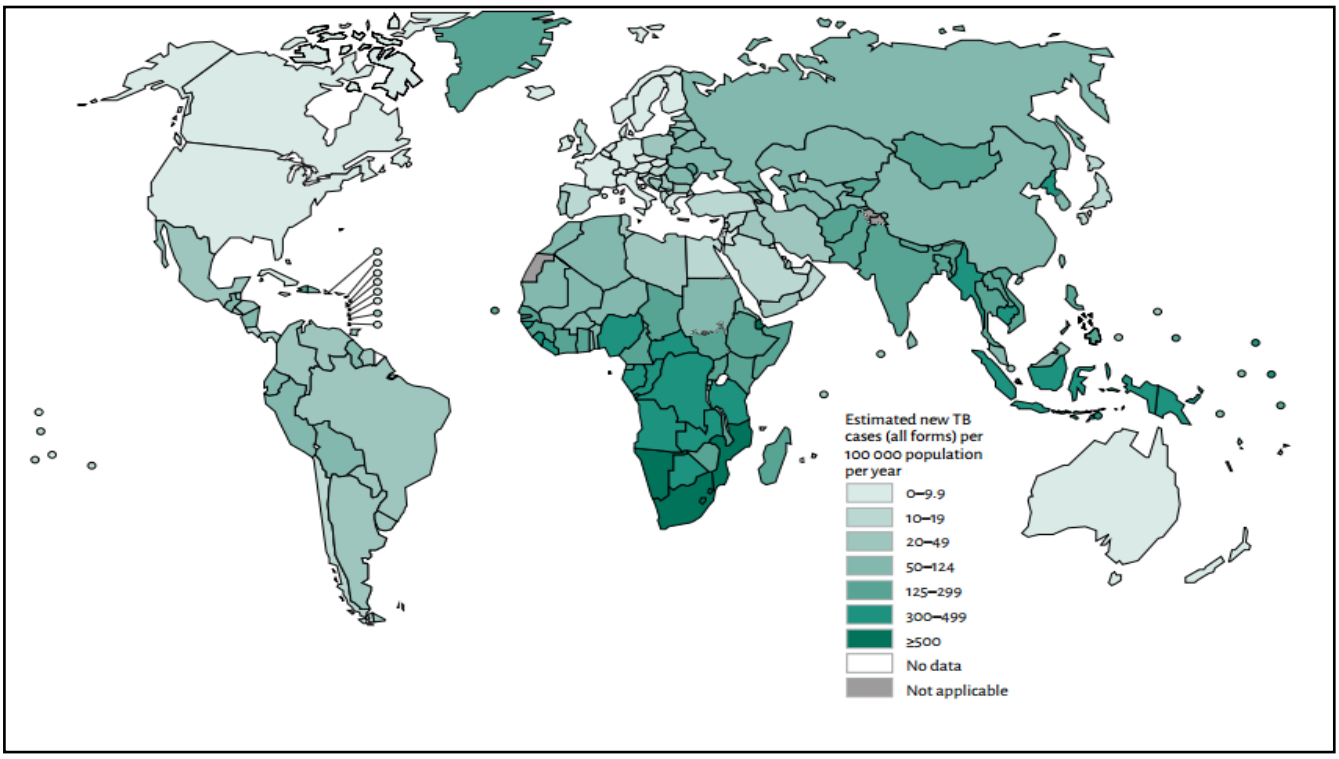


Figure 1.1 Estimated number of new TB cases in 2014 worldwide (WHO Tuberculosis Report, 2015).

On the contrary, the high income countries including most countries in Western Europe, the United States of America, Canada, New Zealand, Japan and Australia were recorded the lowest incidence rate (< 10 cases/ 100 000 population per year) (WHO, 2015). Several factors that are related to high TB burden including co-infection with HIV, poverty, homelessness, imprisonment, immigration and ineffective TB control programmes (Joan *et al*., 2010).

### 1.3 TB in Malaysia

TB in Malaysia is still a major health problem and the number one cause of death in the early 1940s and 1950s (Iyawoo, 2004). According to the Ministry of Health Malaysia (MOH), TB cases recorded a steady increase from 2007 to 2012 (Table 1.1). The mortality rate was 5.3 - 5.8 deaths for every 100,000 population (Benedict, 2014). Approximately 16,918 TB cases were recorded and responsible for 1,504 dealths in 2007, and increasing number of TB cases were recorded in 2012 with 22,710 cases and 1,520 deaths. Sabah recorded the highest number of TB cases in 2012 (4,426 cases), followed by Selangor (3,560 cases) and Sarawak (2,430). The influx of immigrants especially from the TB burden country like Indonesia, Myanmar, Cambodia, Vietnam and Philiphines, is one of the contributing factors for the rise in the number of TB cases in the country (Benedict, 2014). MOH also reported that approximately 13.9 % of TB cases in Malaysia were discovered among the immigrant population (MOH, 2012).

| | 2007 | | 2008 | | 2009 | | 2010 | | 2011 | | 2012 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **STATE** | **CASES** | **DEATHS** | **CASES** | **DEATHS** | **CASES** | **DEATHS** | **CASES** | **DEATHS** | **CASES** | **DEATHS** | **CASES** | **DEATHS** |
| PERLIS | 112 | 13 | 129 | 12 | 119 | 18 | 121 | 18 | 141 | 18 | 185 | 13 |
| KEDAH | 812 | 63 | 843 | 61 | 903 | 78 | 1,000 | 101 | 1,084 | 85 | 1,174 | 100 |
| PENANG | 880 | 98 | 943 | 127 | 1,039 | 150 | 1,062 | 148 | 1,126 | 128 | 1,245 | 117 |
| PERAK | 1,210 | 181 | 1,160 | 152 | 1,250 | 179 | 1,389 | 151 | 1,309 | 159 | 1,554 | 190 |
| SELANGOR | 2,484 | 84 | 2,374 | 87 | 2,342 | 100 | 2,829 | 111 | 3,242 | 216 | 3,560 | 108 |
| WPKL/PUTRAJAYA | 1,253 | 22 | 1,387 | 47 | 1,429 | 57 | 1,455 | 33 | 1,907 | 83 | 1,906 | 64 |
| N.SEMBILAN | 395 | 35 | 506 | 44 | 408 | 41 | 426 | 35 | 449 | 35 | 480 | 16 |
| MALACCA | 349 | 58 | 337 | 48 | 339 | 54 | 395 | 32 | 511 | 48 | 546 | 55 |
| JOHOR | 1,591 | 188 | 1,700 | 189 | 1,855 | 180 | 2,058 | 134 | 2,038 | 151 | 2,046 | 182 |
| PAHANG | 723 | 116 | 779 | 109 | 838 | 130 | 806 | 107 | 788 | 132 | 890 | 112 |
| TERENGGANU | 661 | 107 | 730 | 125 | 762 | 125 | 664 | 108 | 667 | 81 | 733 | 114 |
| KELANTAN | 1,265 | 289 | 1,354 | 295 | 1,324 | 205 | 1,333 | 232 | 1,448 | 232 | 1,436 | 140 |
| LABUAN | 45 | 3 | 67 | 3 | 66 | 2 | 80 | 3 | 106 | 1 | 99 | 1 |
| SABAH | 3,433 | 164 | 3,376 | 140 | 3,515 | 179 | 3,728 | 233 | 3,794 | 188 | 4,426 | 245 |
| SARAWAK | 1,705 | 83 | 1,821 | 93 | 1,913 | 84 | 1,991 | 111 | 2,056 | 87 | 2,430 | 63 |
| **TOTAL** | **16,918** | **1,504** | **17,506** | **1,532** | **18,102** | **1,582** | **19,337** | **1,557** | **20,666** | **1,644** | **22,710** | **1,520** |

Table 1.1 TB cases and deaths in Malaysia, 2007 to 2012 (Benedict, 2014).

### 1.4 MTB

Mycobacterium belongs to the Mycobacteriaceae family in the Actinobacteria phylum. MTB is a Gram-positive bacterium. It has straight or slightly curved rod-shaped, non-motile and non-spore forming bacterium. It has 2-4 μm in length and 0.3-0.6 μm in diameter (Iseman, 2000; Todar, 2012). Figure 1.2 shows the image of MTB bacillus under scanning electron micrograph. It has a complex cell wall and composed of glycolipids, peptidoglicans, peptide side-chains and mycolic acids (Todar, 2012). It has 4,411,529 base pairs (bp) genome and 3924 predicted protein-coding sequence (Cole *et al*., 1998). The MTB genome was successfully sequenced and was published in 1998. MTB has a high content of guanine (G) and cytosine (C) in its DNA (65.6%). The high GC content may be one of the survival strategies employed by the bacterium, since stability of DNA increases directly with number of GC bonds (Cole *et al*., 1998).

### 1.5 Symptoms of TB

The most common MTB infection is in the lungs, known as pulmonary TB. People with pulmonary TB have symptoms like cough, weakness, chest pain, bloody sputum (hemoptysis), and may develop severe breathing problems. TB patients also commonly have symptoms such as fever, cough, malaise, night sweats and loss of appetite and weight. TB can also affect other parts of body, including, lymph nodes, kidneys, spine or brain, gastrointestinal tract, bones and others, resulting in extra pulmonary TB (EPTB). The symptoms of EPTB are depending on which area of the body is infected. If TB infected the lymph nodes, it can cause swollen glands and a painless red mass, usually at the sides and base of the neck. It also can cause paralysis if it infected the spinal cord.

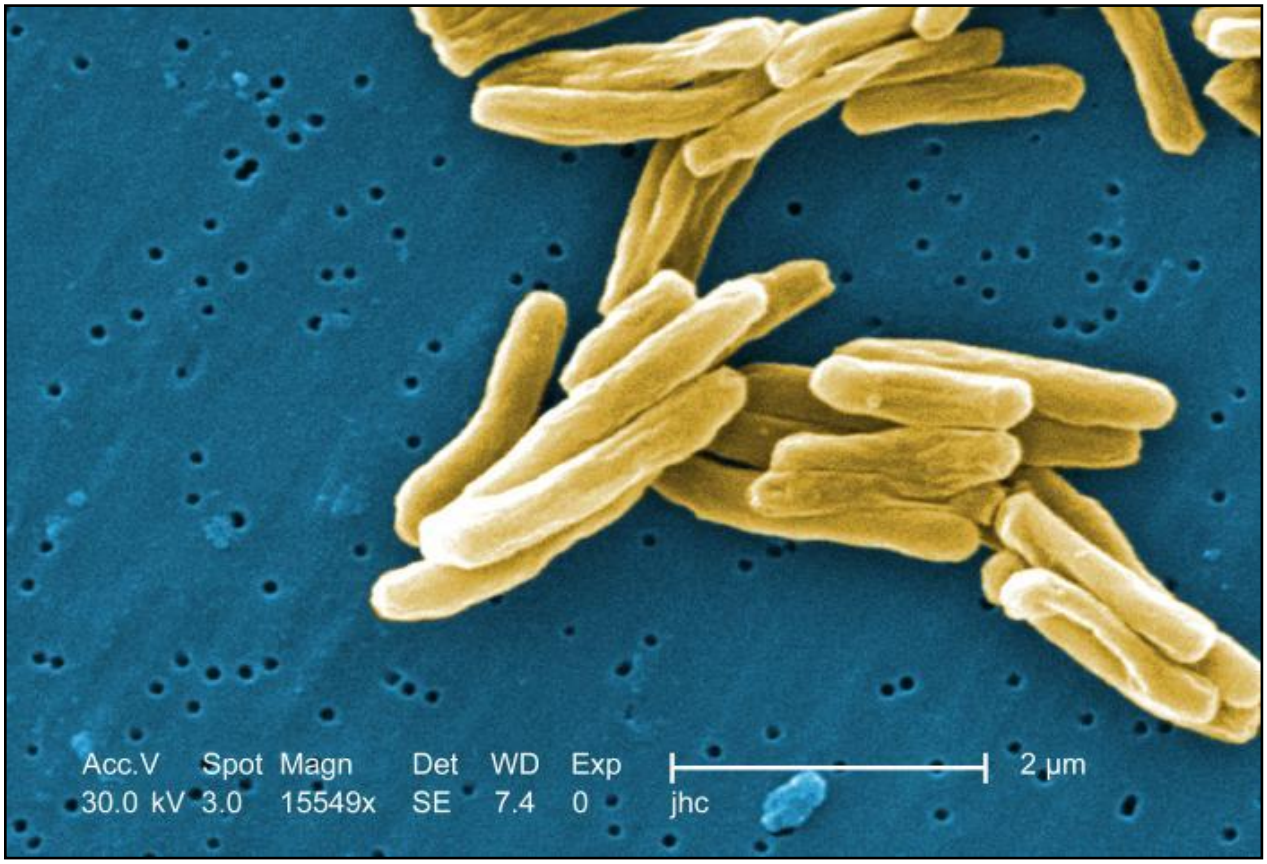


Figure 1.2 Scanning electron micrograph of MTB bacillus.

(The picture was taken from Todar (2012) [Online]. Available from: http://textbookof bacteriology.net/tuberculosis.html).

### 1.6 Transmission and pathogenesis of TB

A deep understanding of the transmission and pathogenic mechanisms of MTB infection and colonization of susceptible hosts is crucial to the vaccine developments and treatments for TB. The most frequent route of MTB infection is via airborne transmission. TB can spread to other people through coughing, sneezing, talking or singing when the person inhales air containing the droplet nuclei (McNerney *et al*., 2012). The droplet nuclei of the bacteria entering the body through mouth or nasal passages, upper respiratory tract, bronchi and reach the alveoli of the lungs. A cascade of host defense mechanisms is triggered when a small number of inhaled MTB reach the terminal airspaces of the lungs and are ingested by alveolar macrophages (Orme, 2014).

In lungs, MTB infects macrophages, B- and T-lymphocytes, natural killer cells (NK), neutrophils, dendritic cells (DCs), γδ T cells and other cells to initiate the formation of granuloma (Figure 1.3). The granuloma may provide a local environment for interactions of innate and adaptive immune system components (Pitt *et al*., 2013). Infection does not necessarily lead to TB disease, only 3-10 % of infected individuals will develop the disease during their life-time, while more than 90 % of the infected individuals sustain the bacteria in latent state. Bacteria inside the granuloma can become dormant or latent, resulting in LTBI. The lifetime risk for immunocompetent individuals is estimated at around 5-10 %, whereas the risk of reactivation for immunocompromised, HIV-infected individual is around 10 % annually (Smith & Ross, 1994).

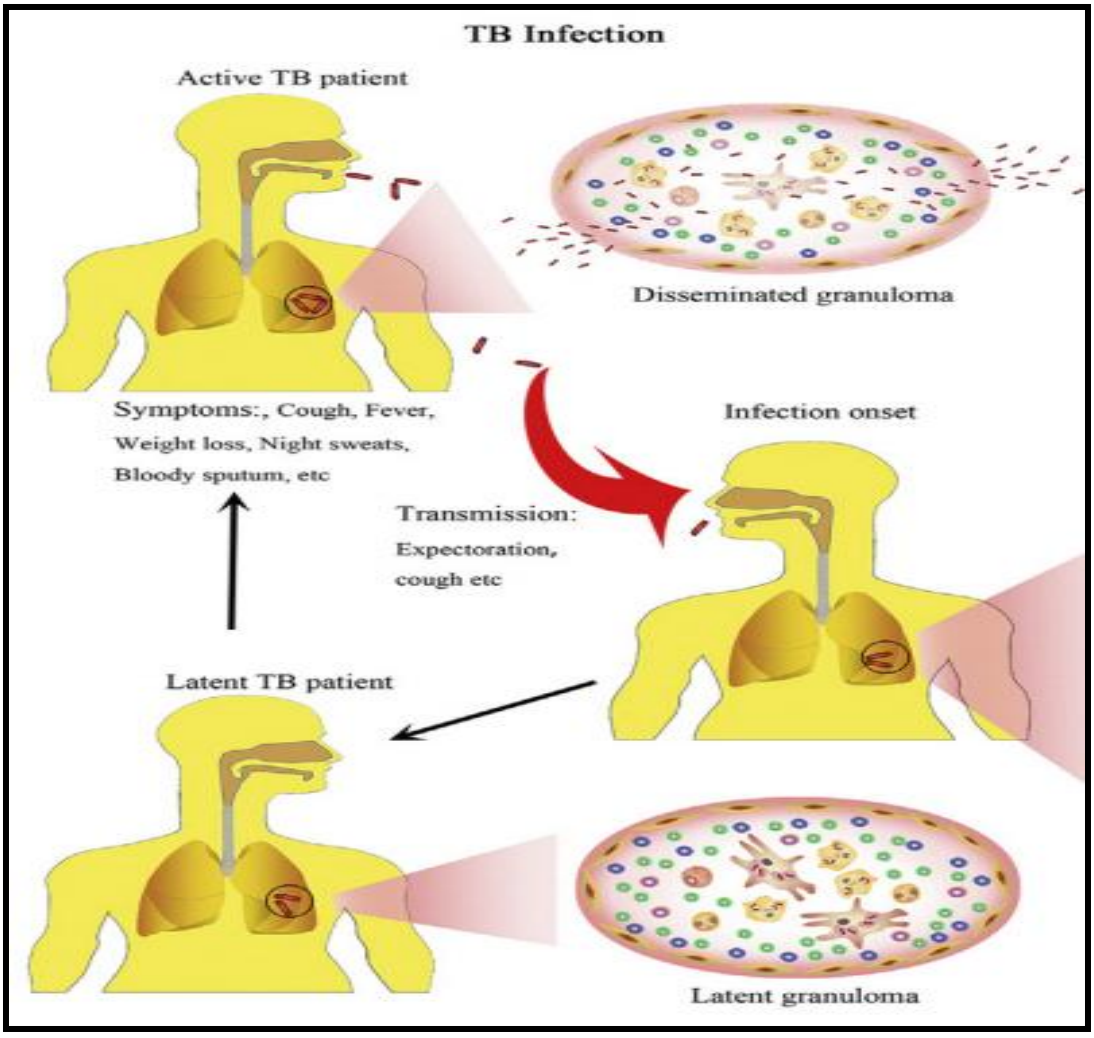


Figure 1.3 Diagram of TB infection and transmission (Tang *et al*., 2016).

### 1.7 Immunity to TB

The immune response is composed of innate and adaptive immune response that are responsible to maintain immune homeostasis against any infections. The interaction between MTB and host cells is complex because MTB is sophisticated mycobacteria and can survive inside host cells (Coler *et al*., 2001).

#### 1.7.1 Innate immunity

Innate immune response is a form of natural immunity in which the immune cells have never encountered the pathogen, but can nevertheless eliminate it. Innate immune system comprised of the anatomical barriers, the complement system, macrophages, DCs, granulocytes and NK cells. The innate response mainly controlled by macrophages and neutrophils. Macrophages are involved in phagocytosis of MTB and kill the bacteria by production of reactive oxygen and nitrogen species. Other cells, neutrophils are recruited into the infection / inflammation sites when inflammatory signals are triggered (Urban, 2006).

NK cells are large granular CD3−CD56+ lymphocytes, constituting approximately 10 % of peripheral blood lymphocytes. The vast majority of NK cells (90-95 %) are cytotoxic and do not produce Interferon-gamma (IFN-γ). Only 5-10 % of NK cells are IFN-γ producing cells. It can mediate protection through elimination of MTB infected cells and by secretion of cytokines that activate the adaptive immune response. It is also involved in optimizing the capacity of CD8+ T cells to produce IFN-γ and to lyse infected cells (Vankayalapati & Barnes, 2009).

Another important step in host innate responses is the recognition of MTB products on macrophage and DCs by Toll-like receptors (TLR) (Belvin & Anderson, 1996; Medzhitov *et al*., 1997; Visintin *et al*., 2001). There are four TLRs involved in recognition of MTB namely TLR2, TLR4, TLR8 and TLR9 (Quesniaux *et al*., 2004; Zhang *et al*., 2004). TLR2 is involved in detection of the mycobacterial glycolipids like lipoarabinomannan (LAM) (Means *et al*., 1999b; Underhill *et al*., 1999) and MTB lipoprotein, 19 kDa (Brightbill *et al*., 1999; Noss *et al*., 2001). TLR4 functions in inflammatory responses in chronic TB infections (Fremond *et al*., 2003).

### 1.7.2 Adaptive immunity

#### 1.7.2.1 Cellular immune response

Cell mediated immune response is important to control MTB infection. CD4+ T cells and CD8+ T cells are two important subsets of T cells to control TB. CD4+ T cells recognize antigens via Major Histocompatibility Complex (MHC) class II molecules, while CD8+ T cells recognize antigens presented by MHC class I. CD4+ T cells play an important role in removing infected APC, while CD8+ T cells can directly eliminate MTB infected cells and intracellular pathogen (Silva *et al*., 2000; Tang *et al*., 2016). Type 1 CD4+ helper T (Th1) cells secrete IFN-γ, Tumor Necrosis Factor-α (TNF-α) and Interleukin-2 (IL-2) to recruit and activate T cells (Prezzemolo *et al*., 2014). In turn, the cytokines will react with activated macrophages in upregulation of inducible nitric oxide synthase, which leads to the production of ROI such as nitric oxide (NO) and RNI ($O_2$). IFN-γ is the signature cytokine of the Th1 T cell and play role in macrophage activation. It can activate macrophages to become more bactericidal, producing greater quantities of ROI (Kaufmann, 2002).

CD8+ T cells play important roles in the control of MTB infection through two pathways of cytolysis. Firstly, CD8+ T cells release perforin and granzymes (serine protease) to lyse the infected cells (Vanja *et al*., 2002). Secondly, CD8+ T cells lyse the target cells via Fas/FasL interaction, resulting in apoptosis or lysis of the target cells (Vanja *et al*., 2002). Another subset of CD4+ T cells, Th17 may produce IL-17, which can trigger the recruitment of neutrophils in control TB (Tang *et al*., 2016). On the other hand, the unconventional γδ T cells and CD1-restricted T cells may also play role in TB control (Tang *et al*., 2016). γδ T cells can recognize the components of MTB such as glycolipid to control TB (Tang *et al*., 2016). CD1 T cells play an important role in protection against microbial pathogens that contain lipids on their cell walls or membranes (Jayawardena *et al*., 2001; Chiu *et al*., 2002; Van *et al*., 2004).

#### 1.7.2.2 Humoral immune response

B cells play important role in the regulation of the host response to MTB. B cells can produce antibodies which could functions in multiple aspects of both innate and adaptive immune response (Figure 1.4). These antibodies can bind specifically to the pathogens and neutralize or eliminate the pathogens from the body (Abbas & Lichtman, 2003). Antibody responses to MTB may have different roles based on the stages of infection and diseases. First, in primary progressive TB, for example in children under two years of age, levels of specific antibodies are low due to the paucibacillary nature of the disease and the associated low antigen burden, to the immaturity of the child's immune system, or to both (Shajo *et al*., 2010).

During latent infection, a small number of tubercle bacilli persist inside granulomas where bacillary multiplication is restricted by the host immune response. During this state, some bacterial proteins may induce measurable levels of antibody. As preclinical disease, some bacterial proteins may induce antibody responses before clinical manifestations appear due to their relative immunodominance and / or increased production. Reactivation tends to be associated with a gradual increase in serum levels of Immunoglubulin (Ig) G antibody, which is detected in at least 90 % of reactivation disease patients (Lyashchenko *et al*., 1998; Shajo *et al*., 2010).

The B cells could also function as APC to interact with T cells (T follicular helper cells) at the germinal centre (GC) (Chan *et al*., 2014). Besides that, B cells may produce the proinflammatory cytokines, TNF-α and IL-6 and reduce production of cytokine IL-10 (Chan *et al*., 2014). Antibodies can also form immune complex that fix complements and modulate the inflammation in infected tissues (Chan *et al*., 2014).

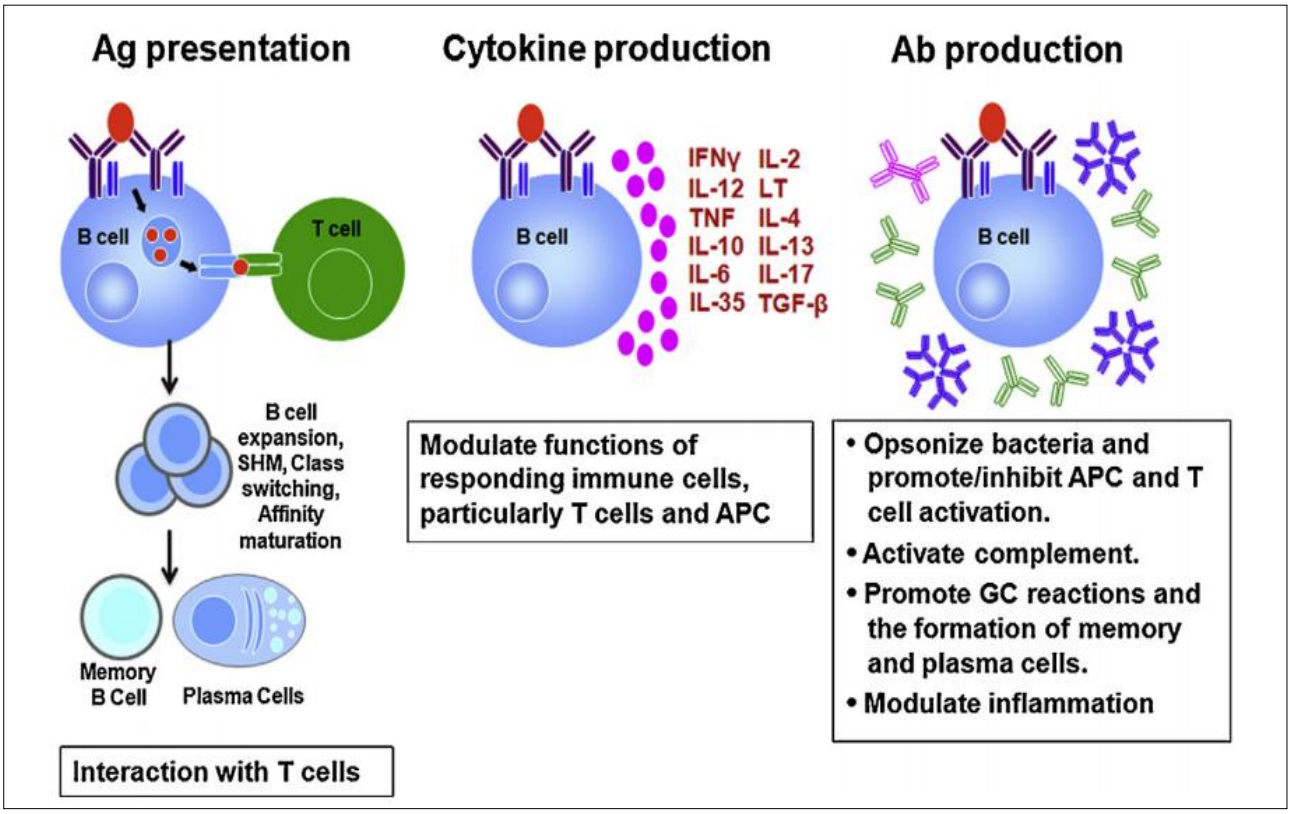


Figure 1.4 Mechanisms by which B cells shape the immune response to MTB (Chan *et al*., 2014).

### 1.8 Control of TB

#### 1.8.1 Diagnosis of TB

Rapid and accurate detection of MTB is very important for TB control. The primary method of examination for TB patient with symptoms is using sputum smear microscopy. However, this method is time consuming and has high contamination rate. According to WHO guideline, the sputum samples must be taken at least two or three slides per patient on successive days (two days) and the results must show positive acid-fast bacilli (AFB) to comfirm the infection (WHO, 2013). To date, microscopic system using TBDx automated system (Signature Mapping Medical Sciences, Herndon, VA, USA) has been implemented in TB diagnosis. This technology will be able to detect and diagnose MTB sputum smear automatically, it save time, more effective and safer than previous diagnosis (Cheon *et al*., 2016). However, the most accurate and sensitive method in TB diagnosis is culture method. It was used as a gold standard technique in diagnosis of active TB. Moreover, there are several new liquid culture system such as Bactec MGIT 960 (BD Diagnostics, USA) and BacT/Alert MB (bioMerieux, France). These systems have higher sensitivity and decrease time of diagnosis compared to the conventional method (Cheon *et al*., 2016).

The additional diagnosis such as chest radiograph, Tuberculin skin test (TST) and Interferon-gamma release assays (IGRAs) are used to comfirm the diagnosis of TB. The chest radiograph is additional diagnosis especially for the acute pulmonary TB disease. Primary pulmonary TB typically has a number of abnormalities and reveals hilar lymphadenopathy in the lung (Jeong & Lee, 2008) (Figure 1.5). TST and IGRAs are the widely used methods for diagnosing active TB and LTBI. The TST was performed by

injecting a small amount of purified protein derivative (PPD) into the skin of the forearm. After 48-72 hours of injection, the diameter of induration on the arm is measured (Figure 1.6). If the diameter of induration is larger than or equal to 10 mm, it is considered a positive reaction (Froeschle *et al*., 2002). IGRAs are *in vitro* blood test for detection of MTB antigens. The IGRAs were developed based on immunological mechanisms between host antigen-specific T cells and MTB antigens (ESAT-6, CFP-10), which resulting in secretion of IFN-γ, a pro-inflammatory cytokine (Whitworth *et al*., 2013). Two types of IGRAs are currently available in the cilinical practice for TB diagnosis, namely T-SPOT.*TB* test (Oxford Immunotech, Oxford, UK) and QuantiFERON Gold In-tube (GFT-GIT, Cellestis, Victoria, Australia) (Figure 1.7). For the T-SPOT.*TB* test, the results are based on the measurement of the number of IFN-γ spots obtained, while for GFT-GIT test, the results are based on the concentration of IFN-γ released is determined by ELISA (Cheon *et al*., 2016).

Molecular diagnosis using Nucleic Acid Amplification Test (NAAT) is a sensitive method that can produce a much faster result than conventional methods for diagnosis of TB infection and drug resistance detection (Dheda *et al*., 2013). The most common amplification techniques widely used is Polymerase Chain Reaction (PCR) (WHO, 2006). Besides, there are other commercially available NAAT methods for TB diagnosis such as Xpert MTB/RIF (Cepheid, USA), PURE-TB-LAMP (Eiken Chemical, Japan), and Genotype MTBDR*sl* (Hain Lifescience, Germany) (Cheon *et al*., 2016). The Xpert MTB/RIF is the most advanced tool and was recommended by WHO as initial diagnosis test for patients with HIV or MDR-TB are suspected (Cheon *et al*., 2016).

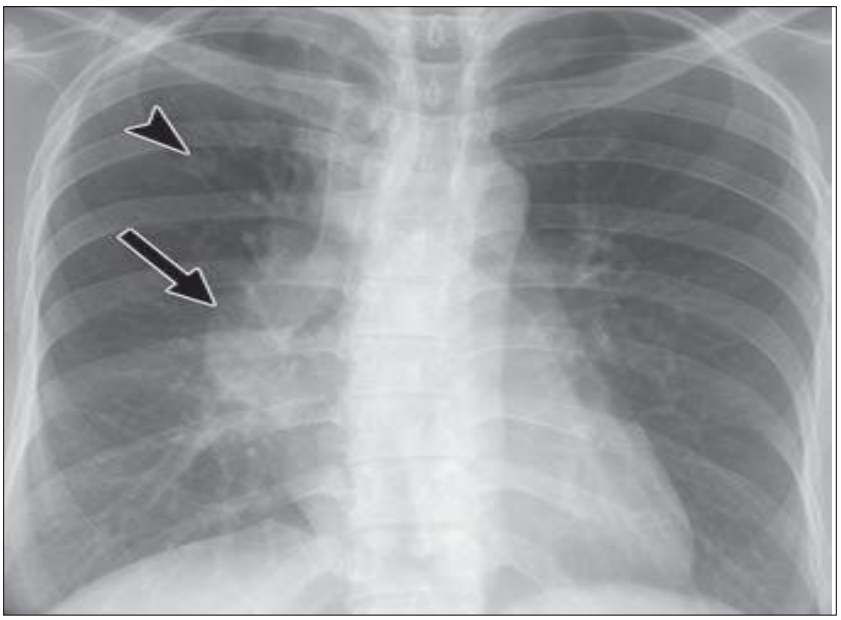

Figure 1.5 Primary TB manifesting primarily lymphadenopathy. Posteroanterior chest radiograph shows right hilar mass (*arrow*). Note smaller nodule (*arrowhead*) in right upper lung zone (Jeong & Lee, 2008).

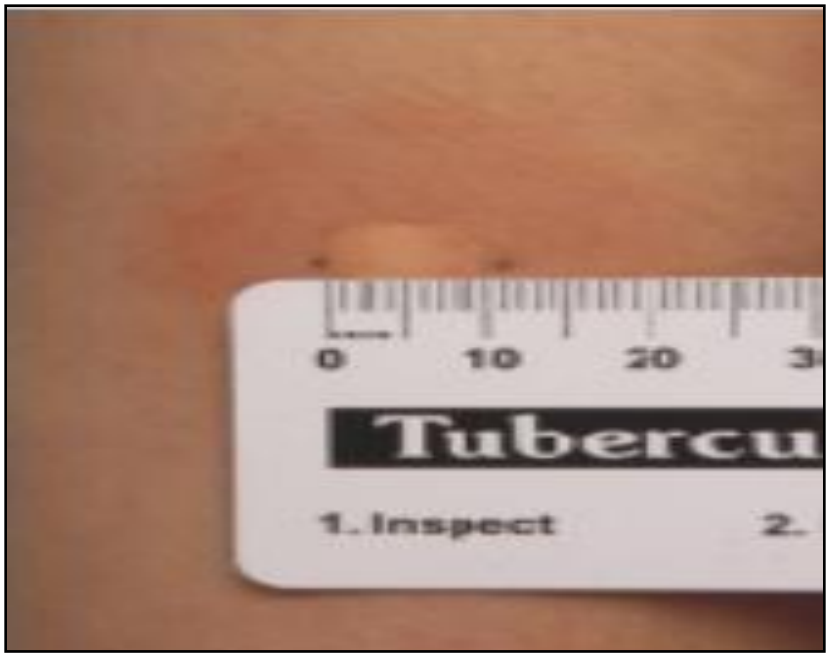


Figure 1.6 TST: The size of induration is measured 48-72 hours after injection (CDC, 2015).

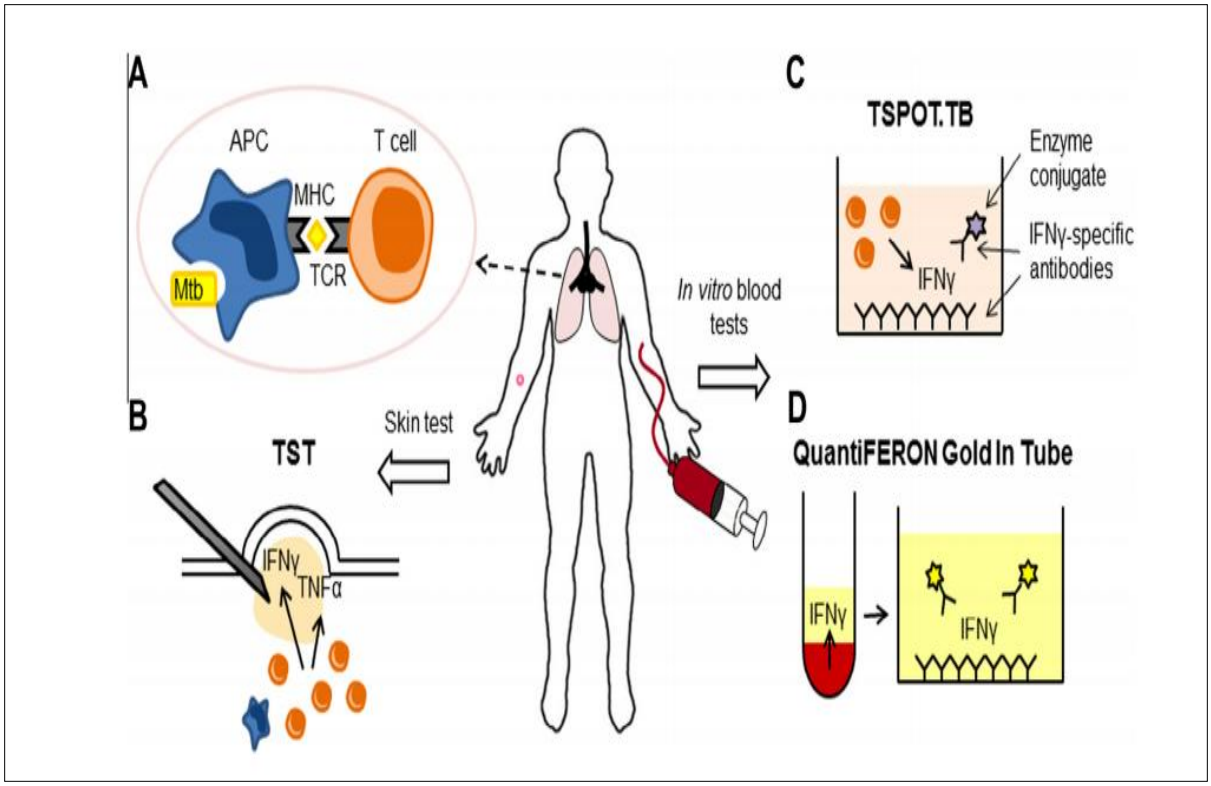


Figure 1.7 Immunological basis of TST and IGRAs. (A) Upon TB infection, presentation of antigens by APCs to priming of antigen-specific T cells. (B) TST used to detect a hypersensitivity reaction *in vivo* following intradermal injection of TB antigens. (C, D) IGRAs detect a T cell IFN-γ response *in vitro* following overnight stimulation of PBMCs or whole blood with TB antigens (Whitworth *et al*., 2013).

### 1.8.2 Treatment of TB

Drug treatment is fundamental for controlling TB. The objectives of treatment are to cure TB patients and to break the transmission of TB as well as to prevent of a possible relapse. Drug treatment started in 1944 with streptomycin (SM) and paraaminosalicylic acid (PAS). In 1952, isoniazid (INH) was combined to the previous drugs, resulting in improving the efficacy of treatment but the treatment still administered for 18-24 months. In 1960, ethambutol (ETH) was introduced to substitute PAS, and the treatment was shortened to 18 months. Then, rifampicin (RIF) was introduced in the 1970's into the combinations, resulting in the treatment was shortened to just nine months. Finally, in 1980, pyrazinamide (PZA) was introduced into the treatment, which reduced treatment further to only six months (Friedman, 2000).

An initial TB treatment should receive all four drugs namely INH, RIF, PZA and ETH. Two drugs (INH and RIF) should be administered over the course of the six months of treatment, and the other two drugs (PZA and ETH) should be administered for the first two months or until the bacilloscopy results are negative (Caminero *et al*., 2015). The drugs should be taken on an empty stomach in the morning. Noncompliance with the treatment compromises the curing process and induced drug resistance (Caminero *et al*., 2015).

### 1.8.3 Prophylaxis

#### 1.8.3.1 BCG

BCG vaccine was developed more one hundred years ago (between 1908 and 1920) by two French scientists, Albert Calmette and Camille Guerin at the Institute Pasteur in Lille, France. The *M. bovis* strain became attenuated and lost the virulence after underwent 231 serial passages (Mahairas *et al*., 1996; Behr *et al*., 1999; Gordon *et al*., 1999; Brosch *et al*., 2001). In July 1921, the BCG was successfully administered on newborn child with no harmful effect. From then on, BCG was widely used in children and was promoted by WHO in 1974.

BCG is the only licenced TB vaccine that currently used in many countries worldwide. The BCG protection is greater when it is administered to neonates and children (up to 80 %), but only 50 % of adults are protected (Colditz *et al*., 1994; Dietrich *et al*., 2003). There are several factors that contributing to the failure of BCG, such as the absence of important genes in BCG, BCG strains used, the freeze-dried vaccine preparations, the genetic and age variability of the vaccines, the influence of environmental mycobacteria strains, latent MTB infection in vaccinees and the different routes of administration (Colditz *et al*., 1994; Lagranderie *et al*., 1996; Fine *et al*., 1999; Brandt *et al*., 2002; Eddine & Kaufmann 2005; Norazmi *et al*., 2005).

#### 1.8.3.2 Development of new generation vaccines against TB

Several approaches and strategies are applied in developing more effective and safer vaccines than BCG. There are three approaches adopted by vaccine designers; i) replacing the BCG antigen with long-term protection genes; ii) preparing BCG vaccinees

with booster; and iii) developing novel therapeutic TB vaccines. So, there are several vaccine strategies currently developed and are being tried based on these approaches.

#### 1.8.3.2.1 BCG replacements

##### 1.8.3.2.1.1 Recombinant BCG (rBCG) strains

The first rBCG vaccine, rBCG30 was constructed by modification of BCG by inserting of plasmid pMBT30 into BCG (Horwitz *et al*., 2000; Principi & Esposito, 2015). The vaccine was developed in order to over-express the secretion of Ag85B in host immune system. The phase 1 clinical trial completed with good protection without major adverse effects in animal models. However, the vaccine was unable to continue for further development due to an antibiotic resistance problem (Costa *et al*., 2014).

The second recombinant BCG vaccine was VPM1002 (ΔureCHly$^{+}$rBCG) (Principi & Esposito, 2015). Some modifications were made to the BCG genome. The gene encoding for listeriolysin (Hly) of *Listeria monocytogenes* was integrated into the BCG genome, inactivation of urease C (ureC) and the insertion of hygromycin resistance genes. Phase I clinical trial has been completed and the phase IIa clinical trial in infants is ongoing (Grode *et al*., 2013; Principi & Esposito, 2015).

##### 1.8.3.2.1.2 Live attenuated MTB strains

MTBVAC01 was constructed by inactivated of *Pho*P and *fad*D26 genes of MTB strain to increase safety (Perez *et al*., 2011). In preclinical studies demonstrated that it is safe and confer a protection against TB infection (Arbues, 2013). The vaccine is entering phase 1 clinical trial (Principi & Esposito, 2015).

#### 1.8.3.2.2 Booster vaccines

##### 1.8.3.2.2.1 Viral vectored vaccines

MVA85A was constructed by modification of Vaccinia Ankara virus expressing Ag85A, as a heterologous booster for the BCG vaccine (McShane *et al*., 2004). In phase 1 clinical trial it was safe and well tolerated (McShane *et al*., 2004). Phase IIb clinical trial was conducted in South Africa (Tameris *et al*., 2013). Although the trial showed MVA85A was safe, it elicited only moderate immune response and no significant protection against MTB infection (Tameris *et al*., 2013).

Another viral vectored vaccine is Aeras 402 (Crucell Ad35), which contains Ag85A, Ag85B and TB10.4 (Radosevic *et al*., 2007; Capone *et al*., 2013). In phase I clinical trial, Aeras 402 was shown to significantly protect mice and in humans and safely induce CMI responses (Radosevic *et al*., 2007; Abel *et al*., 2010). Phase II clinical trial in children is ongoing (Principi & Esposito, 2015).

##### 1.8.3.2.2.2 Protein-adjuvanted vaccines

M72F is a subunit vaccine containing Ag32A and Ag39A (Leroux-Roels *et al*., 2013). It was delivered with AS01 (liposomes, MPL and QS21) as adjuvant, which induced the highest vaccine-specific responses (Leroux-Roels *et al*., 2013). In phase I/IIa trials, it was safe and stimulates CMI responses (Day *et al*., 2013). Phase IIb clinical trial is ongoing (Tang *et al*., 2016).

Hybrid 1/IC31 is a combination with Hybrid 1 (Ag85B-ESAT-6) and IC31 as adjuvant. Phase I clinical trial was completed and phase IIa clinical trial is ongoing (van Dissel *et al*., 2010; van Dissel *et al*., 2011). HyVac4/Aeras-404 vaccine was constructed by

combination of Ag85B and TB10.4. Phase II clinical trial HyVac4 in adjuvant with IC31 is ongoing (Lee *et al*., 2014). Hybrid 56/AERAS-456 vaccine was generated from the combination of Ag85B-ESAT-6 and Rv2660c. The vaccine shows better protection than H1 and BCG when in adjuvant with CAF01 (Aagard *et al*., 2011). The vaccine is ongoing in phase IIa clinical trial (Tang *et al*., 2016).

### 1.8.4 Therapeutic vaccines

#### 1.8.4.1 RUTI

RUTI is an inactivated vaccine that composed of detoxified, fragmented MTB cells and delivered in liposomes (Cardona *et al*., 2006). The vaccine was used as therapeutic vaccine to shorten the treatment of active TB disease and LTBI (Cardona *et al*., 2006). Phase IIa clinical trial was completed. It was safe and no systemic adverse events and few local side effects consisting of minor pain (Groschel *et al*., 2014).

#### 1.8.4.2 *Mycobacterium vaccae* (Mv)

*Mv* vaccine was generated by heat-killed whole *Mv* become an immunotherapeutic vaccine against TB. The mycobacterium contains antigens such as Hsp71, Hsp65, LAM and 40 kDa secreted antigens (Collins *et al*., 1983). In phase I/II clinical trials, it could boost BCG-primed host immunity and improve clinical symptoms of treatment-naïve TB patients (Vuola *et al*., 2003; Yang *et al*., 2011). Phase III clinical trial is still ongoing (Tang *et al*., 2016).

#### 1.8.4.3 *Mycobacterium indicus pranii* (MIP)

MIP is an immunotherapeutic vaccine that was generated by heat-inactivated whole MIP. It was first developed as a vaccine against leprosy. It also showed protection and induced T cell responses better than BCG in animal models (Gupta *et al*., 2009). Phase III clinical trial was completed in India but the results not yet published (Tang *et al*., 2016).

### 1.9 Reverse Vaccinology (RV)

RV is an approach based on *in silico* prediction of vaccine antigen candidates using genetic sequences rather than the pathogen itself. This method is applied to identify *in silico* the complete repertoire of immunogenic antigens without the need of culturing the microorganism. There are three main approaches that have been applied in mining the whole MTB genome 3989 ORFs products and down-selecting the number of antigens to be tested in animal models as vaccine candidates. The first approach led by Anne de Groot, used an *in silico* screening approach with EpiMatrix consisting of the entire MTB coding genome for human MHC class I and class II epitopes (Groot, 2001). Second approach was concentrated on antigen discovery 16 genomic regions of MTB are deleted or lacking in BCG (Attiyah, 2008). Finally, several studies reported the identification of novel vaccine candidates following *in silico* selection, expression as DNA or recombinant protein, and *in vivo* testing for immunogenicity and protective efficacy (Zvi, 2008).

### 1.10 *Mycobacterium smegmatis* (MS)

MS is a non-pathogenic mycobacterium and has been successfully used as a vaccine adjuvant in the prevention and treatment of MTB (Guo *et al*., 2012). The safety and reliability of vectored vaccine have been documented (Yang *et al*., 2009). MS grows 10 times faster than BCG and the MTB-associated proteins expressed by MS are identical to

the natural MTB proteins in terms of biochemical and immunological properties (Zhang *et al*., 2010). MS is also rapidly destroyed by phagolysosomal protease in the phagosomes of infected cells, helping in rapid uptake of expressed antigens in MS (Luo *et al*., 2000; Kuehnel *et al*., 2001). Single inoculation of the MS live vaccine has been shown to provoke sustained induction of target antigens. rMS live vaccine generated by sub-cloning exogenous genes can be used for the treatment of MTB infection. The underlying mechanisms of rMS therapy include restoration of protective immunity, increased generation of $H_2O_2$ and nitric oxide by monocyte-macrophage cells, and a shift of the immune response from Th2 type to Th1 type (Yi *et al*., 2007; Garberi *et al*., 2011). These changes may facilitate clearance of MTB, particularly the drug resistant MTB (Yi *et al*., 2007; Garberi *et al*., 2011).

**1.11 LAA**

There are about 50 LAA which belong to the DosR regulon, which control the expression of these proteins during latency (Singh, 2014). Many of them are believed to be good T cell antigens, but the functions of most of their encoded proteins are still unknown. Their functions are categorized into several categories, such as redox balance, metabolism and energy, cell wall and membrane proteins, stress proteins, host-pathogen interactions and hypothetical proteins. In this study, two genes Rv2005c and Rv2031c belong to stress proteins, Rv3130c belongs to redox balance, metabolism and energy, and Rv3127 belongs to nitrogen metabolism (Singh, 2014). Many studies performed to investigate the potential LAA as vaccine candidates. The most common LAA used as vaccine candidates is Rv2031c (*Hsp*X or α-crystallin). It can induce both active and latent infection and generates humoral and CMI response (Singh, 2014).