PROTEOLIPOSOMES FROM *Mycobacterium bovis* BCG AND *Mycobacterium smegmatis* AS NEW VACCINE CANDIDATES AGAINST TUBERCULOSIS

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by

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AIDS	Acquired immunodeficiency syndrome
AL	Aluminum hydroxide gel
ANOVA	One Way Analysis of Variance
AFB	Acid fast bacillus
B.C	Before Century
BCG	Bacille Calmette Guerin
CD	Cluster of differentiation
CFU	Colony forming unit
CFP	Culture filtrate protein
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DOC	sodium deoxycholate
DTH	Delayed type hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
ESAT-6	6-kDa Early Secreted Antigenic Target
ETH	Ethambuthol
γδ	Gamma delta
HIV	Human Immunodeficiency Virus
HLA	Human Leukocytes Antigen
HRP	Horseradish Peroxidase
IFN-γ	Interferon- γ
IgG	Immunoglobulin G
IGRAs	Interferon- γ Release Assays
INH	Isoniazid
IL	Interleukin
kDa	kilo Dalton
LTBI	Latent TB infection
MDR-TB	Multi-drug resistant-Tuberculosis
MHC	Major histocompatability complex
MT	Montanide ISA 51 VG

Mtb	*Mycobacterium tuberculosis*
Mtb-CFP	Culture filtrate protein from *Mycobacterium tuberculosis*
Mtb-CW	Cell wall from *Mycobacterium tuberculosis*
Mtb-SCWP	Soluble cell wall protein from *Mycobacterium tuberculosis*
Mtb-WCL	Whole cell lysate from *Mycobacterium tuberculosis*
NAATs	Nucleic acid amplification Test
NCDs	Non-communicable disease
NK	Natural killer
NTM	Non-Tuberculous Mycobacteria
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PLs	Proteoliposomes
PLBCG	Proteoliposomes from BCG
PLMs	Proteoliposomes from *Mycobacterium smegmatis*
PPD	Purified protein derivative
PZA	Pyrazinamide
QFT-G	Quantiferon TB Gold
QFT-GIT	Quantiferon TB Gold In Tube
rBCG	Recombinant bacilli Calmette Guerin
RD	Region of difference
RIF	Rifampin
RT	Room temperature
SDS PAGE	Sodium Dedocyl Sulphate-Polyacrylamide Gel Electrophoresis
SEC	Size exclusion chromatography
SEM	Standard error mean
TB	Tuberculosis
TEM	Transmission electron microscopy
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TST	Tuberculin skin test
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant-Tuberculosis

PROTEOLIPOSOMES DARIPADA *Mycobacterium bovis* BCG DAN *Mycobacterium smegmatis* SEBAGAI CALON-CALON VAKSIN YANG BAHARU TERHADAP TUBERCULOSIS

ABSTRAK

Tuberculosis (TB) adalah disebabkan oleh *Mycobacterium tuberculosis* (Mtb). Penyakit ini adalah jangkitan yang boleh membawa maut dan ia merupakan salah satu daripada punca utama kematian dan penyakit di dunia. Bakteria ini biasanya menyerang paru-paru dan anggota badan yang lain seperti otak, buah pinggang dan tulang belakang. Bacille Calmette-Gúerin (BCG) merupakan vaksin yang digunakan secara meluas di seluruh dunia dan ia merupakan satu-satunya vaksin yang digunakan untuk mencegah TB. Kajian menunjukkan BCG dapat melindungi atau sekurang-kurangnya mencegah TB sistemik yang teruk pada kanak-kanak, terutamanya meningitis TB. Walau bagaimanapun, BCG kurang atau tidak dapat memberi perlindungan terhadap penyakit TB pulmonari pada orang dewasa. Maka, calon vaksin yang lebih efektif berbanding BCG adalah sangat diperlukan untuk mencegah penyakit TB serta penularannya. Dengan mengambil kira persamaan genomik and antigenik di antara Mtb dan bakteria tidak patogenik seperti BCG dan *Mycobacterium smegmatis* (Ms), kami menghasilkan proteoliposomes (PLs) daripada BCG dan Ms sebagai calon vaksin terhadap penyakit TB. Kajian bioinformatik yang telah dilakukan dan keputusannya menunjukkan kewujudan epitope-epitop sel T dan B di dalam PLs daripada BCG dan Ms yang serupa dengan yang dihasilkan secara *in vivo* oleh Mtb semasa peringkat jangkitan. Keputusan penentuan antigen leukosit manusia (HLA) telah menunjukkan epitop-epitop ini

memberikan peratusan yang terbaik dalam populasi Melayu dan Cuba. Oleh sebab itu, PLs daripada BCG (PLBCG) dan Ms (PLMs) telah dihasilkan untuk menilai keimunogenan dan tindak balas reaktif silang terhadap antigen-antigen daripada Mtb. PLs ini telah disahkan berbentuk sfera dan mempunyai sifat yang sama dari segi saiz dan bentuk di bawah pemerhatian mikroskop elektron dan kajian kromatografi. Pada peringkat seterusnya, kajian antigenisiti terhadap PLBCG and PLMs di dalam manusia telah dilakukan. Keputusan menunjukkan individu-individu yang sihat telah menghasilkan IFN-γ, yang mungkin disebabkan oleh imunisasi BCG dan pendedahan kepada mikrobakteria persekitaran. Sebaliknya, pesakit TB menunjukkan tindak balas penghasilan IFN-γ yang rendah terhadap PLs, ini berkemungkinan disebabkan oleh kelemahan sistem imun dan faktor-faktor genetik. Tambahan pula, keputusan esei imunoserapan berkaitan enzim (ELISA) dan analisa pemblotan Western, menunjukkan sera daripada pesakit TB dapat bertindak balas dengan protein-protein yang terdapat di dalam PLs. Ini menunjukkan bahawa PLs mengandungi antigen-antigen yang diekspres secara *in vivo* ketika peringkat jangkitan TB yang aktif. Kajian haiwan pula menunjukkan bahawa mencit yang diimunisasi PLBCG yang digabungkan dengan adjuvan alum (AL) telah menghasilkan tindak balas imun humoral yang spesifik dengan mecetuskan tindak balas campuran Th1 dan Th2 serta tindak balas selular yang spesifik secara *in vivo* melalui tindak balas hipersensitiviti jenis lewat (DTH) yang positif terhadap PLBCG. Imunisasi mencit dengan kombinasi PLBCG dan Montanide ISA 51 VG (MT) telah mencetuskan jenis tindak balas Th2. Kumpulan mencit yang diimunisasi PLMs dengan kombinasi AL telah menghasilkan tindak balas antibodi yang spesifik dengan mencetuskan bentuk tindak balas Th2. Splenosit daripada mencit PLMs tanpa adjuvan telah menghasilkan tindak balas Th2 dalam penghasilan sitokin secara *in*

vitro. Kedua-dua kumpulan mencit PLBCG dan PLMs diadjuvankan dengan alum dan kombinasi PLBCG dengan adjuvan MT telah menghasilkan tindak balas reaktif silang humoral terhadap antigen-antigen Mtb serta menghasilkan bentuk tidak balas Th2 dan gagal menghasilkan sitokin terhadap antigen daripada Mtb. Mencit yang diimunisasikan dengan PLMs diadjuvankan dengan AL telah menghasilkan tindak balas reaktif silang selular yang signifikan secara *in vivo* terhadap lisat keseluruhan sel daripada Mtb (Mtb-WCL). Pengenalpastian protein-protein daripada PLs dan Mtb oleh kumpulan mencit-mencit ini adalah berbeza bergantung kepada jenis adjuvan yang digunakan. Secara kesimpulannya, keputusan-keputusan yang diperolehi daripada kajian ini menyokong pembangunan keupayaan perlindungan PLs terhadap jangkitan Mtb di dalam model haiwan pada masa depan.

PROTEOLIPOSOMES FROM *Mycobacterium bovis* BCG AND *Mycobacterium smegmatis* AS NEW VACCINE CANDIDATES AGAINST TUBERCULOSIS

ABSTRACT

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb). It is an infection that can be fatal and one of the leading causes of death and illness in the world. The bacteria typi cally attack the lungs, but can also attack other parts of the body such brain, kidney and spine. Bacille Calmette-Gúerin (BCG) is the world's most widely used vaccine and is the only vaccine available against TB. Studies show that BCG can protect against, or at least ameliorate, severe forms of systemic TB in children, particularly meningitis. Nevertheless, it seems to be of low or no protective value against pulmonary TB in adults. Thus, candidate vaccines more potent than BCG are desperately needed to protect against TB disease and transmission. Considering the genomic and antigenic homology between Mtb and non-pathogenic BCG and *Mycobacterium smegmatis* (Ms), we decided to evaluate the potential of proteoliposomes (PLs) from BCG and Ms as vaccine candidates against TB. Bioinformatics studies predicted the presence of T and B cell epitopes in PLs from BCG and Ms which are expressed *in vivo* by Mtb during infection. Determination of human leukocyte antigen (HLA) have shown these epitopes provide the best percentage in the population of Malay and Cuba. Therefore, PLs from BCG (PLBCG) and Ms (PLMs) were prepared to evaluate the immunogenicity and cross-reactivity with Mtb antigens. These PLs were confirmed to be spherical and homogenous in their size and shape under electron microscopy and chromatographic study. We subsequently, evaluated the antigenicity of PLBCG and PLMs in humans. We showed that healthy individuals produced IFN-γ in the presence of PLs which

could be explained due to BCG vaccination and contact with environmental mycobacteria. In contrast, the lower response of IFN-γ of TB patients could be explained by immunodepression and genetic factors/susceptability. In addition, ELISA and Western blot analyses showed that the PLs are recognized by sera from TB patients, which suggested the presence of Mtb antigens expressed *in vivo* during active infection in PLBCG and PLMs. Animal studies demonstrated that immunization with PLBCG adjuvanted with alum (AL) induced a specific humoral immune response with elicitation of a mixed Th1/ Th2 pattern and stimulation of specific cellular immune response *in vivo* with positive delayed type hypersensitivity (DTH) response against PLBCG. Immunization with PLBCG combined with Montanide ISA 51 VG (MT) elicited a humoral Th2 immune response against PLBCG. Mice immunized with PLMs adjuvanted with AL showed a specific antibody response against PLMs with elicitation of Th2 pattern. Splenocytes from PLMs- immunized mice produced a Th2 pattern of cytokine response *in vitro* against PLMs. Mice immunized with both PLBCG and PLMs adjuvanted with AL and PLBCG with MT induced a humoral cross-reactive response against Mtb antigens with elicitation of a Th2 pattern but failed to produce cytokine response against Mtb antigens. Mice immunized with PLMs adjuvanted with AL produced cellular cross-reactive responses *in vivo* with significant increase of DTH response against whole cell lysate from Mtb (Mtb-WCL). The pattern of recognition of proteins from PLs and Mtb by immunized animals differs depending of the adjuvant used. In conclusion, the results obtained in the current study support the future evaluation of the protective capability of these formulations in challenge experiments with Mtb in animal models.

CHAPTER 1

INTRODUCTION

1.1 Tuberculosis (TB)

1.1.1 History of TB

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb) that may infect any part of the human body, but most commonly occurs in the lungs. It was recognized in ancient and historical times and may have killed millions of people compared to other microbial pathogen. The earliest cases of TB infection were reported in relics from ancient Egypt, India, and China (Zink *et al.*, 2007). Additionally, evidence of spinal TB features has been identified in Egyptian mummies dating back to 9000 Before Century (B.C) (Hershkovitz *et al.*, 2008). Analysis of 483 pre-Columbian skeletons in Chile showed that about 2% of their community had TB correlated with bony TB (Arriaza *et al.*, 1995). Subsequently, molecular studies have established the presence of Mtb complex DNA in ancient bony specimens (Zink *et al*., 2007; Hershkovitz *et al*., 2008; Donoghue *et al*., 2011).

In the Hippocrates era, around 460 B.C, TB was known as phthisis, the most widespread disease during that time. Phthisis attacked young adults between the age of 18 to 35 and is usually fatal (Frith, 2014). It is believed that phthisis was a hereditary disease but not an infectious disease because the infection commonly happened across the entire family (Daniel, 2000; Frith, 2014).

In the 18th century TB, known as 'White plague', became an epidemic in Europe and resulted in millions of deaths. Then, in the early 19th century, TB is named Scrofula and was also known as "king's evil" because they believed that the kings of England

and France could cure scrofula simply by touching those affected (Wheeler, 2003; Frith, 2014). Generally, scrofula is an infection in the lymph nodes, known as lymphadenitis and it is the term used for lymphadenopathy of the neck. It can be caused by Mtb or atypical mycobacterial (Amir, 2010; Cruz-Knight and Blake-Gumbs, 2013).

In March 24, 1882, tubercle bacilli, the causative organism for TB, were discovered by Robert Koch (Sakula, 1982; Lawn and Zumla, 2011) using methylene blue staining (Sakula, 1982). After the discovery, the Sanatorium era begun and in 1982 the first World TB Day was announced by WHO.

1.1.2 TB incidence

Globally, TB is the second infectious disease that caused the most death in human after Human Immunodeficiency Virus (HIV)/ Acquired immunodeficiency syndrome (AIDS). In 2013, approximatey 9 million people were infected with TB. The incidence was observed in both developing and industrialized countries. It has reported by WHO that an estimated 45% of TB mortality rate and 41% of prevalence rate have declined since 1990 to 2013 (WHO, 2014). This means that the world is on track in reducing TB incidence and the trend needs to be sustained to achieve the TB-related United Nations' Millenium Development Goals (MDG) to narrow down the spread of the TB disease by 2015 (WHO, 2013).

TB incidence has declined in 6 regions in the world where Europe is the fastest region to show the decline at approximately 6.5% per year (Figure 1.1). Eastern Mediterranean and South-East Asia showed the slowest decline compared to other

regions, at less than 1% and 2% per year respectively (WHO, 2013). In 2012, almost 8.6 million TB cases were reported worldwide at a rate of 122 cases per 100 000 population (WHO, 2013) (Figure 1.1). Asia (58%) showed the highest number of TB cases compared to other regions, followed by Africa (27%). The highest TB cases in Asia were reported in India, followed by China, South Africa, Indonesia and Pakistan (Figure 1.2). The other 4 regions showed smaller number of TB cases; 8% in Eastern Mediterranean, 4% in Europe and 3% in the Americas.

In 2012, the incidence of TB increased significantly in areas with high rate of HIV infection (1.0–1.2 million), particularly in Africa (37%) (WHO, 2013). Additional factors also contributed to the high TB burden including Multi-drug resistant-Tuberculosis (MDR-TB), Extensively Drug-Resistant Tuberculosis (XDR-TB), homelessness and poverty, increasing number of refugees, reduction of governmental support in TB prevention and lack of treatment programs (WHO, 2013). Another reason for the rising rate of TB cases is due to the high rate of immigration from countries with a high TB incidence (Abubakar *et al.*, 2011; Pareek *et al.*, 2011).

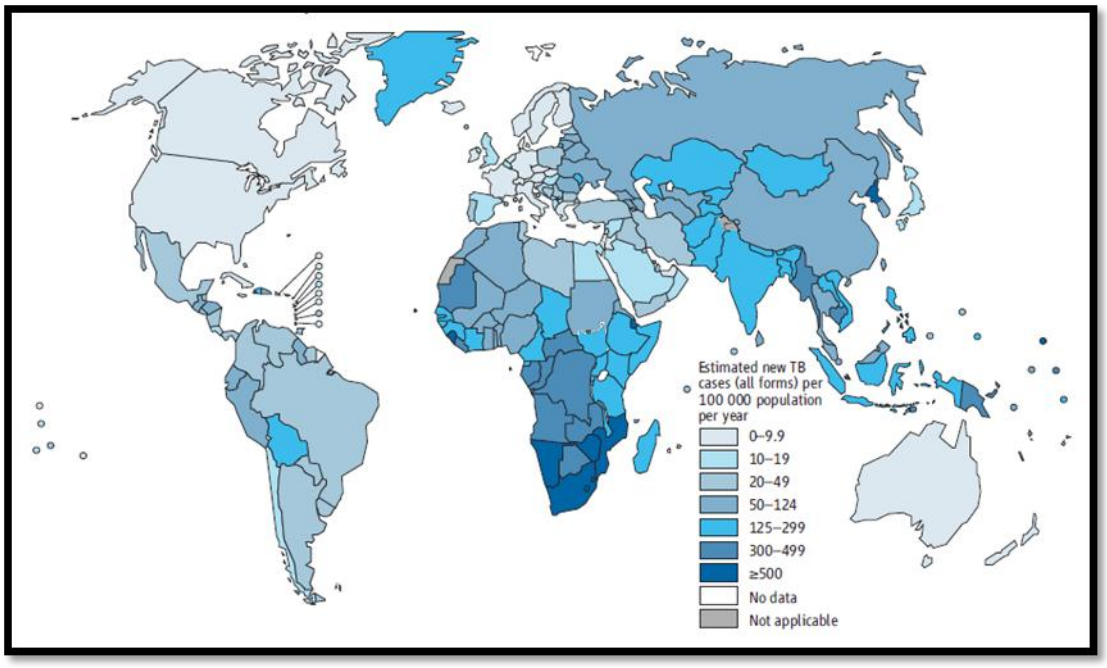

Figure 1.1: TB incidence rates in 2012. (WHO Global Tuberculosis Report, 2013)

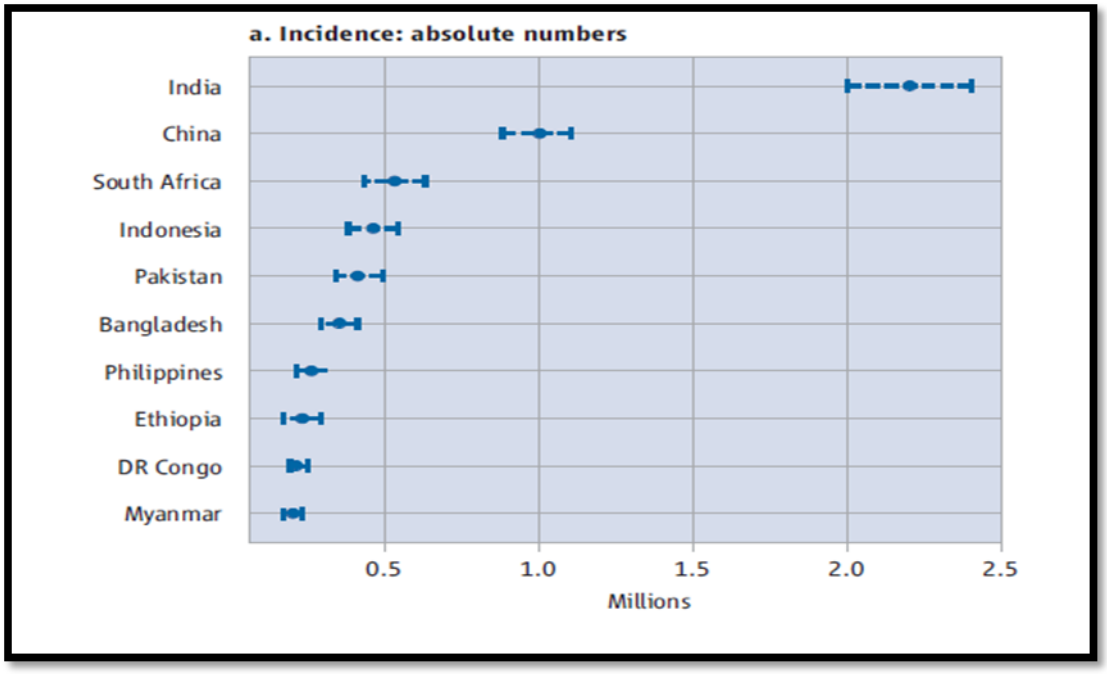

Figure 1.2: TB incidence in top-ten countries, 2012 (WHO Global Tuberculosis Report, 2013)

1.1.3 Mycobacterium tuberculosis

Mycobacterium tuberculosis (Mtb) is a species from the Mycobacteriaceae family and belongs to a distinctive genus of Mycobacterium (Figure 1.3). It is a pathogenic bacterium and the causative agent for TB which was discovered by Robert Koch in 1882. It has a complex cell wall structure which hyropobic and waxy; composed of proteins intermingled in a matrix of peptidoglycan, rich mycolic acid, lipids and carbohydrates (Brennan *et al*., 2003) (Figure 1.4). They are Gram-positive aerobic bacteria, non-motile, non-spore forming, straight or slightly curved rod-shaped microbes, 1-4 μm in length and between 0.3-0.6 μm in diameter (Iseman, 2000; Lawn and Zumla, 2011). Mtb contains granules and vacuoles and grows very slowly with a doubling time of 18-24 hours.

The genome of Mtb is 4,411,529 base pairs (bp) with 3924 predicted protein-coding sequences. The Mtb genome was sequenced and published in 1998 (Cole *et al.*, 1998; Ismael *et al*., 2004). 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).

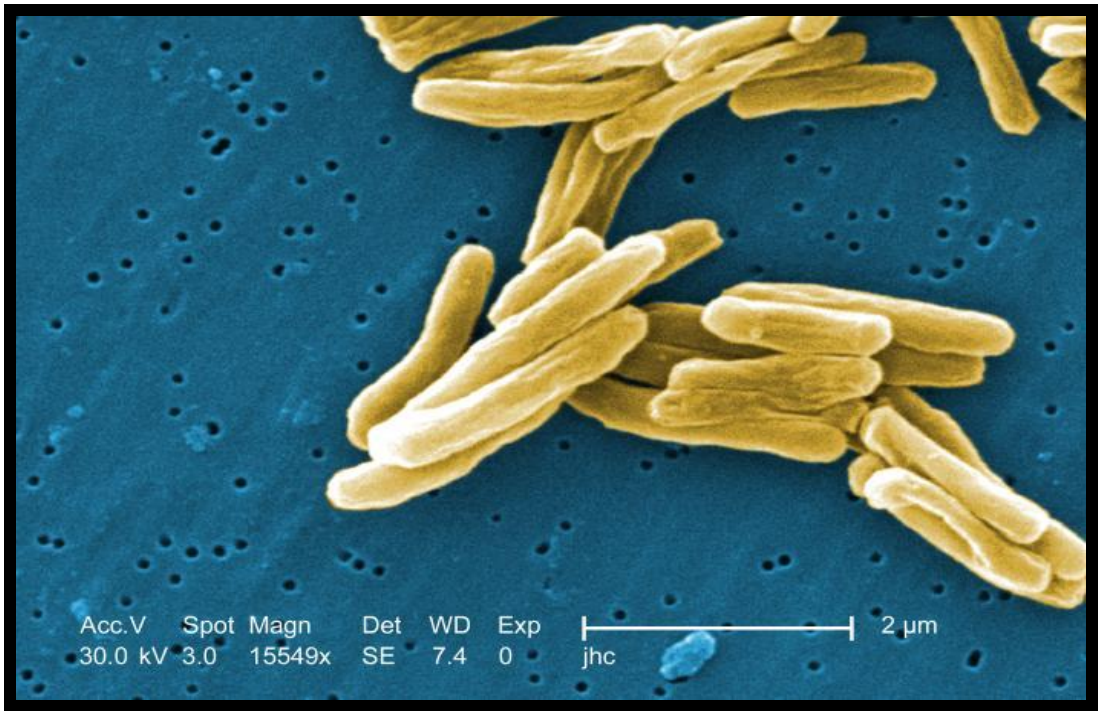

Figure 1.3: Scanning electron microscope of Mtb (under 15549x magnification) (Centers for Disease Dontrol and Prevention, CDC).

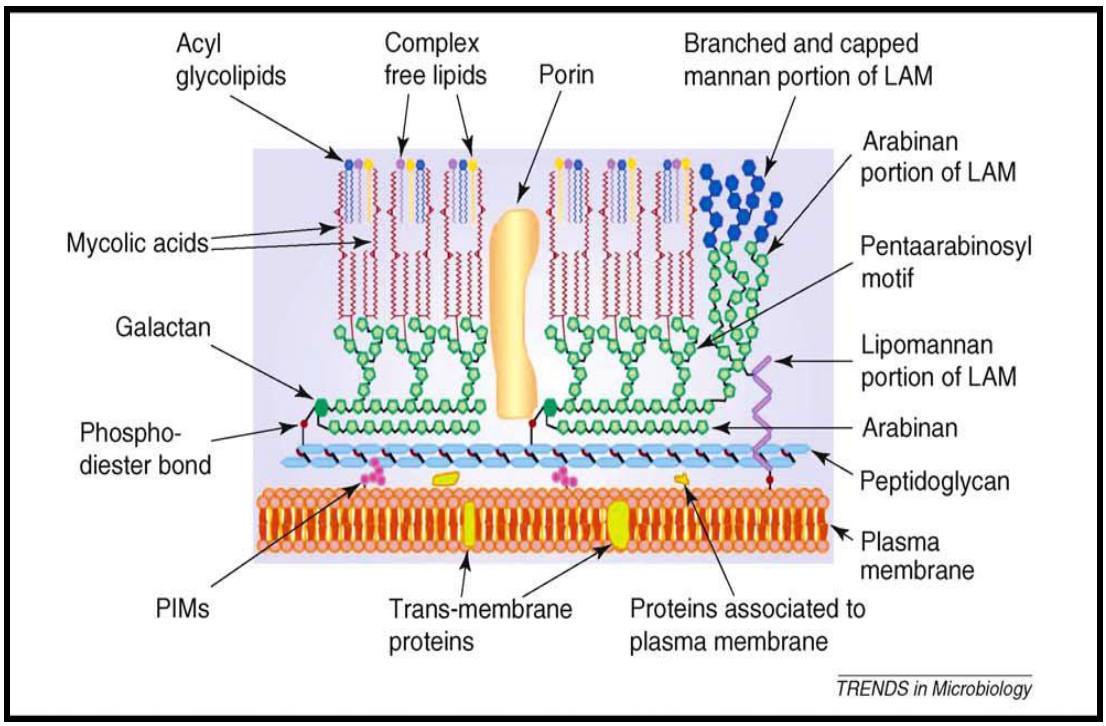

Figure 1.4: Schematic representation of the mycobacterial cell wall (Gokhale *et al.*, 2007).

1.1.4 Transmission and pathogenesis

Airborne transmission is the most frequent route of Mtb infection (Figure 1.5). Although, it can be transmited through the gut, genitourinary tract and eye, or via compromised skin. Mtb is expelled through the airway by a person who have pulmonary or laryngeal TB and is carried in droplets in the air (McNerney *et al.*, 2012), called droplet nuclei (1-5 μm in diameter) which are very light and can remain in air for several hours depending on the environment (McNerney *et al.*, 2012). These bacteria traverse the mouth or nasal passages, upper respiratory tract and bronchi to reach the alveoli of the lungs. Once the smaller droplet nuclei reach the alveoli, the organisms are taken up by alveolar macrophages; some of them are killed and a few multiply in the alveoli, which then enter the bloodstream and spread throughout the body (Russell *et al.*, 2010; Orme, 2014) (Figure 1.5). Mtb will reach any part of the body and rapidly develop into either active disease or remain dormant without causing any symptoms (Schluger and Rom, 1998; Frieden and Driver, 2003). Infection does not necessarily lead to TB disease; in fact, it was reported in several studies, that only 3–10% of immunocompetent individuals that are infected will develop the disease during their life-time (Lawn and Zumla, 2011; Zumla, 2011), while more than 90% of infected subjects sustain infection in a subclinical stage known as latent TB infection (LTBI), in which the pathogen remains in a quiesce nt state (McNerney *et al.*, 2012). LTBI may turn to TB disease by a re-activation process (Figure 1.6). A person with LTBI has a 5-10% chance of developing active TB in their lifetime during the first 5 years after becoming infected (Harries, and Dye,2006; WHO, 2015). Re-activation starts when the immune system becomes weakened and the dormant pathogen become active to cause TB disease.

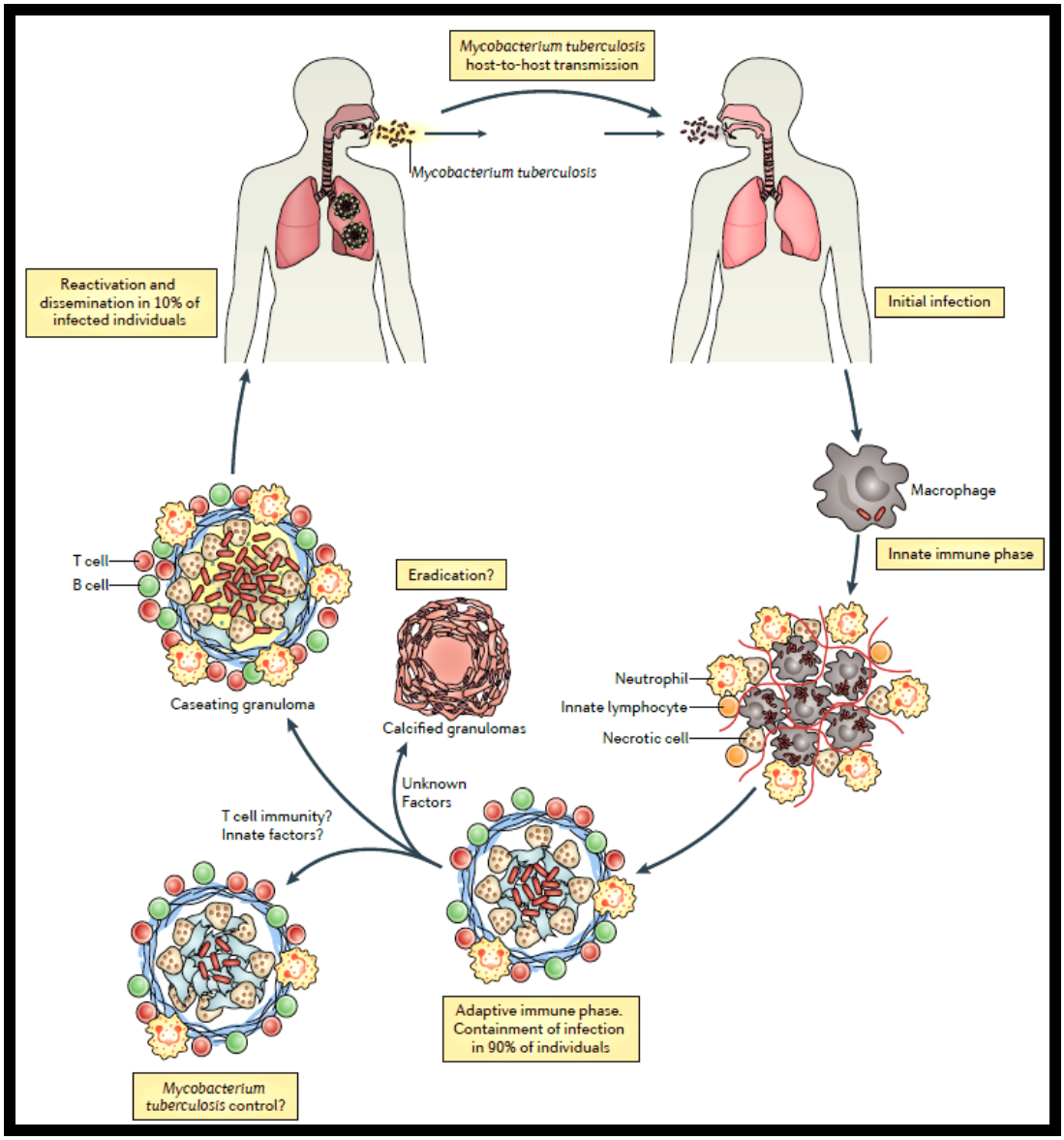

Figure 1.5: Summary of TB transmission and pathogenesis (Nunes-Alves *et al.*, 2014)

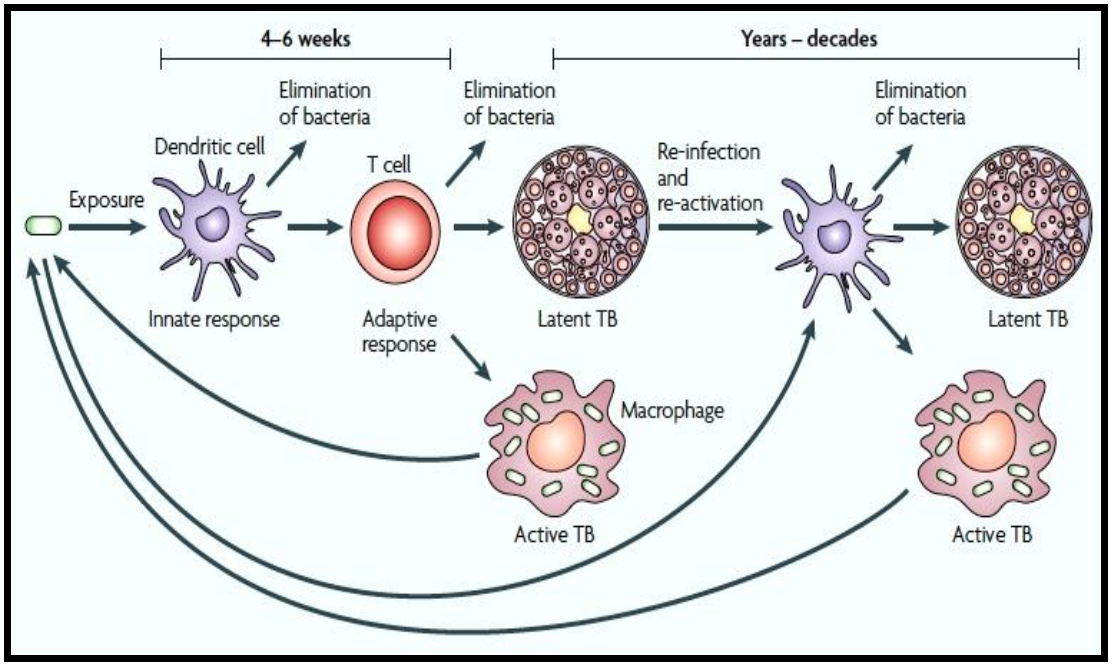

Figure 1.6: The choronolgy of Mtb infection (Young *et al*., 2008).

1.1.5 Symptoms of TB

People with LTBI do not have symptoms because they are in inactive state of the disease. People with TB disease may have any or all of the following symptoms; cough, fatigue, loss of appetite, weight loss, fever and night sweats. As the disease progresses, infected individuals may cough up blood (hemoptysis) and develop severe breathing problems. The symptoms of extra-pulmonary TB vary widely depending on which area of the body is infected. Thus, if TB affects the lymph nodes, it can cause swollen glands and a painless red mass, usually at the sides and base of the neck. When bone is infected, the spine is typically involved which causes back pain lasting weeks to months. Sometimes, the disease can progress to the spinal cord, causing paralysis. Mtb can also infect the joints, with slow progressive swelling and pain, usually in a large joint such as the knee.

1.1.6 TB in children

In 2012, approximately 0.5 million TB cases occurred in children from the 8.6 million TB incidence worldwide. From an estimated 6% of global TB incidence (530 000) in 2012, 8% (74 000) of the incidence caused death among HIV-negative children (WHO, 2013). WHO estimated that TB incidence among children occurs in those younger than 15 years old. Childhood TB appears to be increasing in many parts of the world (Nelson and Wells, 2004b). In many industrialized countries, this situation is associated with new immigration patterns (Nelson and Wells, 2004a; Nelson *et al.*, 2004; Marais, 2014). This trend is associated with many factors including poor economic situation and poor public health infrastructure, rising rates of HIV infection, difficulty of diagnosing childhood TB and the difficulty of diagnosing HIV-associated pulmonary infections in children (Raviglione *et al*., 2012; WHO, 2013).

An estimated 90% of cases in children progressed to serious TB due to primary infection before the age of 2 within the first 12 months without significant symptoms. Primary infection between the age 2 and 10 rarely progressed to serious TB, and disease progression is associated with significant clinical symptoms. Children older than 3 years who show TB symptoms should be clinically diagnosed before serious disease progression. Frequently, children with primary infection after 10 years of age are more likely to progress to adult-type disease (Centers for Disease Control and Prevention). However, early effective intervention in this group will reduce the burden of caveating disease and associated disease transmission in the community.

1.1.7 TB in Malaysia

TB is the most common infectious disease with the highest number of deaths and remains as a major health problem in Malaysia. Moreover, it is the second biggest killer worldwide after Acquired immunodeficiency syndrome (AIDS) (WHO, 2013). As a middle-income country, the number of estimated TB cases continues to rise with rates of TB incidence and mortality of 82 and 8.5 cases per 100 000 population, respectively. In Malaysia, the number of TB cases detected annually is relatively higher than the reported incidence of HIV. About 9% of TB cases are being diagnosed among people living with HIV/AIDS (WHO, 2011).

The Ministry of Health reported an average of 18 500 TB cases between 2007 and 2010 while in 2011, 20 666 new TB cases were registered (MOH, 2011). Pulmonary TB is the most common infectious disease in Malaysia. However, extra pulmonary TB showed an increase and has spread to the entire community every year. In 2011, 2.7% of TB cases involved children of ≤14 years old, while 12.3% involved those of ≥65 years old. The majority (69.5%) of TB patients are between 21-60 years age.

Among the 15 states, Sabah recorded 143.7 cases/ 100 000 population which is the highest in the country, followed by Wilayah Persekutuan (121.7 cases/ 100 000 population), Sarawak (87.4 cases/ 100 000 population), Pulau Pinang (68.7 cases/ 100 000 population) and Kelantan (55.5 cases/ 100 000 population) (MOH, 2011). Approximately 13.9% of TB cases in Malaysia were discovered among the immigrant population (MOH, 2011).

TB incidence has not significantly reduced because of the complexities surrounding the diagnosis, management, and treatment of TB, co-infection with communicable disease such as HIV/AIDS and non-communicable diseases (NCDs) (e.g. diabetes mellitus), challenges related to the treatment of drug-resistant Mtb strains, poverty, illiteracy and smoking (MOH, 2012). In Malaysia, the incidence of TB-HIV in 1990 was six cases and it reached to 933 cases in the year 2002 (MOH, 2012). Additionally, it was reported that diabetes mellitus is the leading risk factor for TB in Malaysia. Diabetic patients are three times more likely to develop TB (Dooley and Chaisson, 2009).

1.1.8 Risk factors

1.1.8(a) Human Immunodeficiency Virus (HIV)

WHO has declared that TB is the number two killer after HIV/AIDS. One in ten people infected with TB (but who are not infected with HIV) becomes ill or at least infected with TB once during his lifetime. Immunocompromised individuals such as HIV patients are at greater risk of developing active TB disease, 12 – 20 times higher than normal individuals. In 2012, 8.6 million people were infected with TB while 1.3 million people died from TB: there were 320 000 deaths in HIV-infected people and estimated 170 000 deaths were from MDR-TB (WHO, 2013). Three-quarters of total TB deaths occurred in Africa and Southeast Asia regions, in which South Africa and India accounted for one third of the global TB deaths (Lawn and Zumla, 2011; Zumla, 2013).

1.1.8(b) Non-communicable diseases (NCDs)

While TB has dominated the number of death worldwide, NCDs including diabetes mellitus, diseases related to smoking and alcoholism, chronic respiratory and cardiovascular diseases, and also mental disorders grew concomitantly as associated diseases with increased number of TB case (Marais *et al.*, 2013; Lönnroth *et al.*, 2010a).

NCDs are one of the risk factors for TB, mainly for the development of the disease as early as infection due to the the negative impact of NCDs on the host defense (Sopori, 2002; Wang *et al.*, 2002; Cegielski and McMurray, 2004). Additionally, NCDs may make difficulties in management and treatment of TB, due to clinical challenges among people with diabetes mellitus and behavioral challenges among those who suffer from alcohol use disorders (Rehm *et al.*, 2009; WHO, 2009). Moreover, TB can trigger or aggravate NCDs. For example, TB can worsen glucose control and lead to diabetes mellitus among those at high risk groups (WHO, 2009). TB, although not a classical risk factor for chronic respiratory disease, is one of the main causes of lung sequelae and bronchiectasis. TB also identified as an independent risk factor for chronic respiratory disease in a recent review (Salvi and Barnes, 2009).

Lönnroth *et al*. (2008) concluded that heavy drinkers consuming ~0.40 g alcohol a day and/or person who have a bad habit of alcohol use have three times risk for getting TB. A person with low to moderate drinking of alcohol has a lower risk to develop TB (Lönnroth *et al*. (2008). This association seems to be a causal factor for TB progression (Rehm *et al.*, 2009). Additionally, alcohol has been showed a direct

toxic response on the immune system. Several studies recommend that excessive alcohol consumption can weaken cell-mediated immunity and macrophage functions, which is important for the host response to Mtb infection (Szabo, 1997; Cegielski and McMurray, 2004; Rehm *et al.*, 2009). Moreover, previous studies have demonstrated that heavy alcohol consumption may be one of cause of the malnutrition deficiencies, which can also weaken immunity (Gonzalez-Reimers *et al.*, 2008; Knudsen *et al.*, 2014), which can also weaken immunity (Porretta *et al.*, 2012).

Globally, severe malnutrition in parts of the developing world causes a large increase in the risk of developing active TB (Cegielski and McMurray, 2004; Lönnroth *et al.*, 2010b; Dye *et al.*, 2011; Cegielski *et al.*, 2012), due to its damaging effect on the immune system. Along with overcrowding, poor nutrition may also contribute to the strong link observed between TB and poverty.

1.1.9 Immune response against Mtb

1.1.9(a) Innate immune response

Host immune response plays an important role in the defense against Mtb during primary infection (Cooper *et al*., 2011). The component of the innate immune system comprised the anatomical barriers, as well as the complement system and several types of innate immune cells, including various leukocytes: macrophage, dendritic cells (DCs), granulocytes and natural killer (NK) cells (Janeway *et al*., 2001; Quintin *et al*., 2014).

Infection of Mtb begins with the inhalation of small droplet nuclei containing Mtb into the pulmonary alveoli. When Mtb enters the alveolar macrophage, Mtb are phagocytised by DCs, alveolar type II pneumocytes and monocyte-derived macrophage (Van Crevel *et al*., 2002; Hossain and Norazmi, 2013; Quintin *et al.*, 2014).

Another crucial step in an effective host innate response is the recognition of Mtb or mycobacterial products on macrophages and DCs by toll-like receptor (TLR) *(*Belvin and Anderson, 1996; Medzhitov *et al*., 1997; Visintin *et al*., 2001). In mammals, TLR is an important connection between the innate and adaptive immune systems, and act as linkers for the recruitment and activation of the adaptive immune system (Werling *et al*., 2003; Akira *et al*., 2004; Pasare *et al*., 2005; Kanczkowskial *et al*., 2007) in host defence against microbial pathogens, particularly Mtb (Kulchavenya, 2013). There are four TLRs involved in recognition of Mtb namely, TLR2, TLR4, TLR9 and possibly TLR8 (Tapping and Tobias, 2003; Bulut *et al*., 2005; Davila *et al*., 2008).

The mycobacterial glycolipids, including lipoarabinomannan (LM) and phosphatidylinositol-mannosides (PIM), are the main factors that activate TLR2 (Ferwerda *et al*., 2005). Several mycobacterial antigens, such as the 19-kilo Dalton (kDa) lipoprotein, activate the innate immune response through TLR2 (Brightbill *et al.*, 1999). In addition, several antigens such Hsp 60, 65, 70 and 38 kDa glycoprotein are reported to activate TLR4 (Jung *et al.*, 2006; Jo *et al.*, 2007; Kleinnijenhuis *et al.*, 2011). A study by Bhatt and Salgame (2007) demonstrated that TLR2 is a central protein for *in vitro* activation of the innate immune response. In addition, TLR2 was

shown to influence the maintenance of Th17 cells in the lung of Mtb-infected animals (Teixeira-Coelho *et al*., 2011) and granuloma formation (Drennan *et al*., 2004; Teixeira-Coelho *et al*., 2011), but not bacterial burden (Sugawara *et al*., 2003; Drennan *et al*., 2004; Bafica *et al*., 2005; Teixeira-Coelho *et al*., 2011). In addition, another important TLR member, TLR8 was suggested to have a potential role in TB-susceptibility in male TB patients and in macrophage immune responses to BCG infection (Davila *et al*., 2008).

Host recognition of Mtb or mycobacterial products will lead to immune cell activation and production of specific cytokines. IFN-γ is the central cytokine and plays a key role in the Th1 development leading to host defence against Mtb infection (Belosevic *et al*., 1989; Sher and Coffman, 1992; Gately *et al*., 1998). The importance of IFN-γ was demonstrated by the increased susceptibility to mycobacterial infection of children with IFN-γ receptor (IFN-γR) deficiency (Jouanguy *et al*., 1996; Jouanguy *et al*., 1997) and in IFN-γR knockout mice (Cooper *et al*., 1993; Flynn *et al*., 1993). In humans, IFN-γ alone is insufficient to enhance the ability of macrophages to control Mtb infection. Thus, other cytokines such as Tumor Necrosis Factor (TNF)-α and Interleukins (IL) are synergistically important in controlling the infection.

TNF-α is produced by T cells, macrophages and DCs (Valone *et al*., 1988; Barnes *et al*., 1993; Henderson *et al*., 1997; Ladel *et al*., 1997; Serbina and Flynn, 1999) and plays a key role in granuloma formation. In TB patients, TNF-α is detected at the site of infection (Barnes *et al*., 1993; Law *et al*., 1996; Casarini *et al*., 1999). IL-1β is also detected at the site of infection (Schauf *et al*., 1993; Law *et al*., 1996; Bergeron

et al., 1997). Studies in mice suggested that IL-1β plays an important role in TB eradication (Juffermans *et al*., 2000; Yamada *et al*., 2000). In addition, IL-12 is also one of the crucial cytokines in controlling Mtb infection and is secreted following phagocytosis of Mtb by macrophages (Henderson *et al*., 1997; Ladel *et al*., 1997). The administration of IL-12 DNA can reduce the bacterial numbers in mice with chronic Mtb infection (Lowrie *et al*., 1999). Furthermore, IL-12, IL-18 and IL-15 are also important in controlling Mtb infection by increasing the production of IFN-γ (Berg *et al*., 2002; Berg *et al*., 2003).

1.1.9(b) Adaptive immunity

1.1.9(bi) Antibody responses against Mtb

Humoral immune response is mediated by antibodies that are produced by activated B lymphocytes (plasma cells) and provides the most effective response against extracellular pathogens. These antibodies bind specifically to the antigens and are important elements in protection against infectious diseases (Janeway, 2005). The immune system then neutralizes or eliminates the pathogen from the body through ingestion and degradation of the antibody-antigen complex by phagocytes or by cell lysis (Abbas and Lichtman, 2001).

Recent studies showed that Mtb induced a humoral immune response to various mycobacterial antigens in humans despite being an intracellular pathogen (Steingart *et al*., 2009). Intradermal BCG vaccination elicits two classes of antibodies such as IgG and IgM. These antibodies are directed to several mycobacterial antigens such glycolipid lipoarabinomannan (LAM) which is known as the major cell wall antigen

(Brown *et al*., 2003; de Valliere *et al*., 2005). Moreover, a study by de Valliere *et al.* (2005) revealed that antibody response shows to boost innate and cell mediated immune responses against mycobacteria.

The humoral immune response against mycobacterial antigens vary depending on the phase of infection (Kunnath-Velayudhan and Gennaro, 2011). For example, in the case of persons with LTBI, they do not have active TB and produce antibody to a different repertoire of Mtb antigens compared to those with active TB. Moreover, animal and human studies have demonstrated that increased antibody titres was directly correlated with the level of mycobacterial burden (Achkar *et al*., 2010; Kunnath-Velayudhan *et al*., 2010; Yu *et al*., 2012). The observation of high antibody titer in active TB patients against mycobacterial antigens has supported the disagreement that these antibodies are not protective. However, similar argument was not used to challenge the perception that immunity against TB is typically based on cell and cytokine mediated, since most of TB patients despite having normal T cell function and producing high IFN-γ (Okamoto *et al.*, 2005; Cavalcanti *et al.*, 2012). Interestingly, antibody titers can be used as markers for TB, in the same way that increases of IFN-γ levels indicate the progression of TB (Lin *et al*., 2009; Diel *et al*., 2011). Although not all the antibodies induced by Mtb infection are protective, several reports highlight the potential importance of specific antibodies in the defense against Mtb (Glatman-Freedman *et al.*, 2000; Ciurana *et al.*, 2004; Lopez *et al*., 2009; Olivarez *et al*., 2009; Acosta *et al*., 2010; Acosta *et al*., 2012; Achkar and Casadevall, 2013; Acosta *et al*., 2013; Alvarez *et al*., 2013).

1.1.9(bii) Cellular responses against Mtb

Cell-mediated immune response is important to control Mtb infection. This response provides a major protective immunity to Mtb rather than the humoral responses (Kaufmann, 1995; Feng *et al*., 1999). Cluster of differentiation (CD) 4+ T cells are central to the human immune response to Mtb infection (Boom, 1996), and supported by other T cell subsets such as CD8+ cytolytic T cells (CTL) and unconventional T cells (γδ and CD1+ T cells) (Kaufmann, 2000; Flynn and Chan, 2001; Kaufmann, 2001; Jung *et al*., 2005; Kaufmann, 2013).

CD4+ T cells recognize antigens via major histocompatibility complex (MHC) class II molecules (Dorhoi *et al*., 2012), whereas, CD8+ T cells also contribute to immune protection against TB (Cooper, 2009) by recognition of antigens presented by MHC class I molecules (Dorhoi *et al*., 2012). In general, CD4+ is responsible for directing an immune response by activating effector cells, whereas CD8+ T cells is the killer cells that lyse infected cells by producing various cytokines such as IFN–γ and TNF-α (Schluger and Rom, 1998). Some authors have reported that T cells such as CD4+, CD8+ and γδ T cells are important in controlling TB infection in mice and cows (Cassidy *et al*., 2001; Walravens *et al*., 2002; Vesosky *et al*., 2004).

Activated CD4+ T cells can differentiate into either Th1 cells which secrete specific cytokines IFN–γ and TNF-α, or into Th2 cells secrete IL-4, IL-5, IL-10 and IL-13. The activation of Th1 cells by Mtb antigens resulted in cytokine production, mainly IL-2 and IFN–γ (Huygen, 2014; Montagnani *et al.*, 2014) . These cytokines will activate macrophages and other T cells (CD8+ and γδ T-cell) to provide strong protection against bacterial infection (Grange *et al*., 1995; Feng *et al*., 1999; Flynn

and Chan, 2001; Raupach and Kaufmann, 2001). Th2 cells play important role in immune responses during Mtb infection by counter-regulating Th1 cells and impairing protective immunity against TB (Romagnani, 2005; Keir *et al*., 2008; De Libero and Mori, 2005). Th2 cells also contribute to down modulation of the immune response to the pathogen (Kaufmann, 2013) and to TB reactivation (De Libero and Mori, 2005; Keir *et al*., 2008; Gold *et al*., 2010; Kaufmann, 2013).

There are two major lysis mechanisms of CD8+ T cells in protecting against Mtb. Firstly, CD8+ T cells expressed perforin and granzymes to lyse the infected cells. In humans, CD8+ T cells produce granulysin that is toxic to Mtb which lyse the infected cells (Stenger *et al*., 1997; Stenger *et al*., 1998). Secondly, CD8+ T cells lyses the target cells via the Fas/FasL pathway (Clark *et al*., 1995). In addition to killing of infected cells, CD8+ T cells mediate anti-microbial effector function by secreting related cytokines such IFN–γ and TNF-α (Herbein, 1997). The γδ T cells and CD1+ restricted T cells also produce IFN–γ which activate anti-mycobacterial activities in macrophages and inhibit the mycobacterial growth (Raupach and Kaufmann, 2001) which is supported by TNF-α. Tsukaguchi *et al*. (1995) demonstrated that γδ T cells have the capacity to display cytotoxicity and produce IFN-γ in response to Mtb infected macrophages. A study performed by D'Souza *et al*. (1997) reported that γδ T cells contribute in early stages of TB infection. As the disease progress, apoptosis mediated by Fas/FasL clears the majority of Mtb and causes reactivation of γδ T cells (Li *et al*., 1998). Raja (2004) suggested that γδ T cells are important in patients with LTBI (Flynn and Chan. 2001; North and Jung, 2004). Studies performed in a primate model demonstrated that the number and activity of γδ T cells increased during primary mycobacterial infection and after BCG vaccination (Barnes *et al*., 1992).

The CD1 family (group I CD1 molecules: CD1a, CD1b, CD1c and group II CD1 molecule; CD1d) is the least common T cell subset in human peripheral blood and the lungs. CD1 plays a significant role in protection against microbial pathogens like Mtb that contain unique lipids on their cell walls or membranes (Brigl *et al*., 2004; Cohen, *et al*., 2009). In humans, CD1a, b, and c have been associated with effective immune response to leprosy and mycobacterial infections. Brigl *et al*. (2004) have shown that CD1 expressions are down-regulated following exposure to live Mtb.

1.1.10 Control of TB

1.1.10(a) Diagnosis

The standard TB diagnosis in clinical examination is routinely performed by acid fast bacillus, followed by culture on solid or liquid media for confirmation (Aber *et al*., 1980) as recommended by WHO guidelines (WHO, 2010). The result of Mtb culture can be obtained up to 8 weeks and in 10–20 % of cases the bacillus is not successfully cultured (Pottumarthy *et al*., 1999). If the patient has pulmonary TB by radiographs but no diagnostic sputum sample, then bronchoscopy should be considered to confirm the diagnosis. Rapid and sensitive tools for diagnosis are required due to the increased incidence of TB worldwide particularly in the HIV endemic regions and the length of time required for the classical tests. Currently, rapid molecular tests are being used for the detection of pulmonary TB (Vassall *et al*., 2011; Boyle *et al*., 2014).

There are several new techniques being developed including fluorescent microscopy which used similar principle as conventional light microscopy. This technique

required an ultraviolet light source such as a high-pressure mercury vapour lamp and an acid-fast fluorochrome dye for staining (Steingart *et al*., 2006). The bacilli are then visibly seen as fluorescent green rods. On the other hand, the most rapid liquid culture system used in laboratory is BACTEC 460. The use of BACTEC for culture can decrease the detection time when culturing using solid media (1–2 months to 1–2 weeks) (Enarson and Ait-Khaled, 2003). Safety issues concerning the radioactivity of BACTEC and its cost, however, prevents its widespread application.

The most recent and advanced technologies to diagnose TB are Nucleic acid amplification tests (NAATs) and Interferon-gamma (IFN-γ) assay (IGRAs). NAATs are used following microscopy to distinguish Mtb from other smear-positive mycobacteria. The technology is able to detect small amounts of genetic material (DNA or RNA target sequences) from Mtb due to repetitive amplification of the target sequences (Dinnes *et al*., 2007). If no target organism is present, there will be no amplification. The most common technique used is polymerase chain reaction (PCR) (Ramachandran and Paramasivan, 2003) and a new technique recently developed is Xpert MTB/RIF. On the other hand, IGRAs has been developed to replace the tuberculin skin test (TST) for TB detection of either active TB or latent TB. The assay is based on purified protein derivative (PPD) and region of difference (RD)-1 specific antigens are able to discriminate between Mtb and *Mycobacterium avium* intracellular complex infection (Streeton *et al*., 1998). Two types of valuable commercial IGRAs: Quantiferon-TB Gold and the newer version of Quantiferon-TB Gold In Tube (QFT-GIT), based on ELISA measure the amount of IFN- γ produced in response to specific Mtb antigens [QFT-G: Early secreted Antigenic Target

(ESAT)-6 and CFP-10, QFT-GIT: ESAT-6, CFP-10 TB7.7]; and enzyme-linked immunospot - based T-SPOT.

The assays measure the number of peripheral mononuclear cells that produce IFN-γ after stimulation with ESAT-6 and CFP-10. The antigens in IGRAs are absent in most of non-tuberculous mycobacteria (NTM) and BCG strain (Anderson *et al*., 2000; Mahairas *et al*., 1996; Harboe *et al*., 1996). Although, IGRAs cannot distinguish between active TB and LTBI (Herrera *et al*., 2010), IGRAs results are not confounded by BCG vaccination and less likely to be confounded by exposure to NTM (Lange *et al*., 2009). Thus, IGRAs shows advantages over the TST in identifying human immune response to Mtb, particularly in population with high NTM exposure and general vaccination.

1.1.10(b) Treatment

TB can be treated by chemotherapy and this approach started in the late 1940s with the introduction of streptomycin. The objective of this approach is to eradicate all tubercle bacteria from the patient and to prevent secondary resistant bacteria. Subsequently, several drugs were introduced including p-aminosalicylic acid (PAS) (1946), isoniazid (INH) (1952), cycloserine (1955), kanamycin (1957), rifampin (RIF) (1965), ethionamide (1966), ethambuthol (ETH) (1968) and pyrazinamide (PZA) (1970) (Friedman, 2000). The usual standard drug regiment short course chemotherapy consists of 2 months of INH, RIF, PZA and ETH, 4 months of INH and RIF. The drugs must be taken either daily or 3 times per week and they have to be given under direct observation (DOTs) by health-care staff as recommended by WHO (WHO, 2010).

Regular drug treatment is a pre-requisite for treatment success. However, under certain circumstances, the doctor may have modified the particular needs of individual patients. The great majority of patients can be considered as non-infectious after 2 weeks of effective drug treatment. If the treatment is taken irregularly, there may be serious consequences: the Mtb may become resistant to the drugs which can cause MDR-TB, where TB cannot be cured or TB disease can spread to others leading to death.

ETH is added if MDR-TB is suspected (American Thoracic Society, 1993). ETH inhibits the synthesis of arabinogalactan and interrupts the mycobacterial cell wall synthesis (Mikusova *et al*., 1995). INH is important in inhibiting the synthesis of mycolic acid (Petrini and Hoffner, 1999). PZA plays an important role in the intensive phase of the bacteria. PZA produced specific enzyme to inhibit the fatty acid synthesis and affects the mycobacterial energy production for survival (Mitchinson *et al*, 2005). RIF is an important agent in the treatment of TB and has bactericidal properties against both intracellular and extracellular Mtb (Friedman *et al*., 2000). In the late 1970s, TB drug development stopped as it was assumed that the effective drug and the combination with BCG vaccination programs would lead to the prevention of TB as a public health threat. Unfortunately, not all TB patients received an effective control of TB treatment in certain areas (Gandhi *et al.*, 2006; Tadesse *et al.*, 2011). Nachega and Chaisson (2003) reported that this phenomenon resulted in the MDR-TB defined as Mtb that is resistant to at least two drugs, INH and RIF. Therefore, an effective surveillance is very important in reducing the potential for MDR-TB, and this is one of the essential factors in gaining control of the global TB problem.