THE DEVELOPMENT OF A CANDIDATE TUBERCULOSIS DNA VACCINE EXPRESSING Mtb8.4 AND Ag85B of *Mycobacterium tuberculosis*

by

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DEDICATIONS

This thesis is specially dedicated to:

My beloved husband, Ahmad Zarizi b. Shaari
My sons, Aiman Haris and Aiman Hakimi
My parents, Dr. Azlan and Dr. Kamariah

Thank you for your love, support and patience...
May God bless you all....

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LIST OF ABREVIATIONS

AFB	Acid fast bacillus
αβ	Alpha beta
Ag85	Antigen 85
APCs	Antigen presenting cells
BCG	Bacille Calmette Guerin
β2-m	Beta-2-microglobulin
CMI	Cell mediated immunity
CFU	Colony forming unit
CTL	Cytotoxic T lymphocyte
ddH2O	Deionised distilled water
DTH	Delayed type hypersensitivity
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
γδ	Gamma delta
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
i.m	Intramuscular
i.p	Intraperitoneal
kDa	kilodalton
KO	Knock-out
LB	Luria-bertani
MHC	Major histocompatibility complex
mAbs	Monoclonal antibodies
MDR-TB	Multi drug resistant TB
NAA	Nucleic acid amplification
NK	Natural killer
Nramp	Natural-resistance-associated macrophage protein
O.D	Optical density

PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPD	Purified protein derivative
rBCG	Recombinant bacille Calmette Guerin
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RD	Region of difference
RE	Restriction enzyme
SIV	Simian immunodeficiency virus
SI	Stimulation index
Th	T helper
TAP	Transporter associated protein
TB	Tuberculosis
TNF	Tumor necrosis factor
UV	Ultraviolet
WHO	World Health Organization

THE DEVELOPMENT OF A CANDIDATE TUBERCULOSIS DNA VACCINE EXPRESSING Mtb8.4 and Ag85B of *Mycobacterium tuberculosis*

ABSTRACT

Tuberculosis (TB) is still one of the major health problems worldwide. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, bacille Calmette Guerin (BCG). However, the efficacy of BCG vaccine continues to be debated. Therefore, a more effective vaccine against TB is urgently needed. DNA vaccination is a new approach to the control of infectious agents. In this study, a DNA vaccine encoding the candidate TB antigens Mtb8.4 and Ag85B was developed using assembly PCR. Balb/c mice were immunized intramuscularly with 50 μg of the DNA vaccine, pNMN023, containing the two antigens, in each hindleg. Reactivity against the Ag85B peptides, P1 and P3 as well as Mtb8.4 showed a consistent Th1 type of immune response by virtue of the increased expression of IL-2, IFN-γ and IgG2a. Splenocytes from immunized mice were also found to proliferate more aggressively when stimulated with the antigens compared to the vector alone. In order to improve the vaccine efficacy, a preliminary prime-boost approach was used. Priming with pNMN023 and boosting with recombinant BCG (rBCG) in Balb/c mice was carried out. Flow cytometric intracellular cytokine analyses of splenocytes from mice immunized with the DNA-rBCG prime-boost regime showed that both $CD4^+$ and $CD8^+$ T cells showed an increase in IL-2 and IFN-γ production following stimulation with either antigens at significantly higher levels than those immunized with rBCG-DNA prime-boost. In conclusion, the data obtained from this study suggest that DNA vaccination in combination with the prime-boost approach provide a potential strategy for developing a candidate vaccine against TB.

PEMBANGUNAN CALON DNA VAKSIN TERHADAP TUBERKULOSIS YANG MENGEKSPRESKAN Mtb8.4 dan Ag85B DARIPADA *Mycobacterium tuberculosis*

ABSTRAK

Tuberkulosis (TB) merupakan salah satu penyakit utama di dunia. Satu-satunya vaksin TB yang terdapat pada masa ini ialah strain yang telah dilemahkan iaitu, *Mycobacterium bovis* bacille Calmette-Guerin (BCG). Bagaimanapun, keberkesanan BCG masih diperdebatkan. Oleh itu, vaksin yang lebih efektif terhadap TB sangat diperlukan. Vaksin DNA merupakan salah satu cara untuk mengawal ejen infeksi. Di dalam kajian ini, vaksin DNA yang mengkodkan antigen TB iaitu, antigen Mtb8.4 dan antigen Ag85B telah dibangunkan menggunakan kaedah PCR himpunan. Mencit Balb/c telah diimunisasi intraotot dengan 50 μg vaksin DNA, pNMN023, yang mengandungi kedua-dua antigen. Kereaktifan terhadap peptida Ag85B, P1 dan P3 juga Mtb8.4 telah menunjukkan peningkatan tindakbalas imun jenis Th1 yang konsisten melalui peningkatan pengekspresian IL-2, IFN-γ dan IgG2a. Splenosit dari mencit yang diimunisasi juga didapati menunjukkan peningkatan gerak balas proliferasi apabila dirangsang dengan kedua-dua antigen. Untuk meningkatkan keberkesanan vaksin, kajian awal menggunakan pendekatan '*prime-boost*' telah digunakan. '*Priming*' dengan pNMN023 dan '*boosting*' dengan BCG rekombinan (rBCG) di dalam mencit Balb/c telah dijalankan. Analisis intrasel sitokin dari splenosit mencit yang telah diimunisasi dengan DNA-rBCG menunjukkan peningkatan IL-2 dan IFN-γ kedua-dua sel T $CD4^+$ dan $CD8^+$ apabila dirangsang dengan kedua-dua antigen berbanding mencit yang diimunisasi dengan rBCG-DNA. Sebagai kesimpulan, data yang diperolehi dari kajian ini mencadangkan bahawa vaksin DNA digabungkan dengan kaedah '*prime-boost*' merupakan salah satu kaedah yang berpotensi untuk membangunkan calon vaksin terhadap TB.

CHAPTER 1

LITERATURE REVIEW

1.1 History of tuberculosis

Tuberculosis (TB) in humans is caused by *Mycobacterium tuberculosis* while *M. bovis* causes TB infection in cattle. The Hippocratic Collection compiled around 400 - 350 B.C. recorded the clinical manifestations and epidemiologic features of phthisis (Greek term), the tuberculous process in the lungs was called a 'phyma' (Iseman, 2000). The frequency of unearthed skeletons with apparent tubercular deformities in ancient Egypt suggests that the disease was common among that population. Evidence of bone lesions suggestive of TB in mummies of North America and Egypt confirms the ancient impact of this disease on early civilizations (Nerlich *et al.*, 2000; Rothschild *et al.*, 2001) and further confirmed by the use of molecular-based diagnosis of TB in some ancient Egyption mummies (reviewed by Bedeir, 2004).

During the golden age of Islam, Ibnu Sina described the clinical features and pathology of TB in Arabic scripts (reviewed by Madkour *et al.*, 2004). The discovery of similarly deformed bones in various Neolithic sites in Italy, Denmark, and countries in the Middle East also indicates that TB was found throughout the world approximately 4,000 years ago.

In the 18th century, TB was well established in Europe and had spread to Africa, Asia, South America and Eastern Europe by the end of the 19th century. In 1882, Robert Koch discovered tubercle bacillus as the causative agent of TB. In 1993, due to the emergence of TB incidence worldwide, TB was declared as a 'global emergency' by the World Health

Organization (WHO) and a decade later, the first international conference on 'TB vaccine for the world' was held in Montreal.

1.2 Disease burden

It is estimated that two billion people (one third of the world's population) is infected with *M. tuberculosis* (WHO, 2001), where 8.8 million people will show clinical diseases and 1.5 million will die every year (WHO, 2004). TB also occurs in Southeast Asia with three million new cases every year and a quarter of a million in Eastern Europe (Girard *et al.*, 2005). These situations are worsened with the estimation that only 40% of new cases of pulmonary TB are currently detected (Dye *et al.*, 2002). If controlling efforts are not accelerated, 10 million new TB cases are expected in 2010 (Dye, 2000).

Rising rates of drug-resistant TB have contributed to worsen treatment outcomes in some regions (Figure 1.1). The incidence of TB increased in areas with high rates of human immunodeficiency virus (HIV) infection. Approximately, 14 million people are co-infected with *M. tuberculosis* and HIV, including more than 70% of those living in some regions of sub-Saharan Africa (WHO, 2004).

An initiative to address the increase of TB disease burden known as "Stop TB" was created in 1998 to ensure that endemic countries are adequately supported by technically and financially to control TB (Raviglione & Pio, 2002). Among the supports include the US National Institute for Allergy and Infectious Diseases (NIAID), the Aeras Global TB Vaccine Foundation, the European Union Commission and pharmaceutical manufacturers including GlaxoSmithKline (GSK) and IDRI-Corixa (Hewinson, 2005).

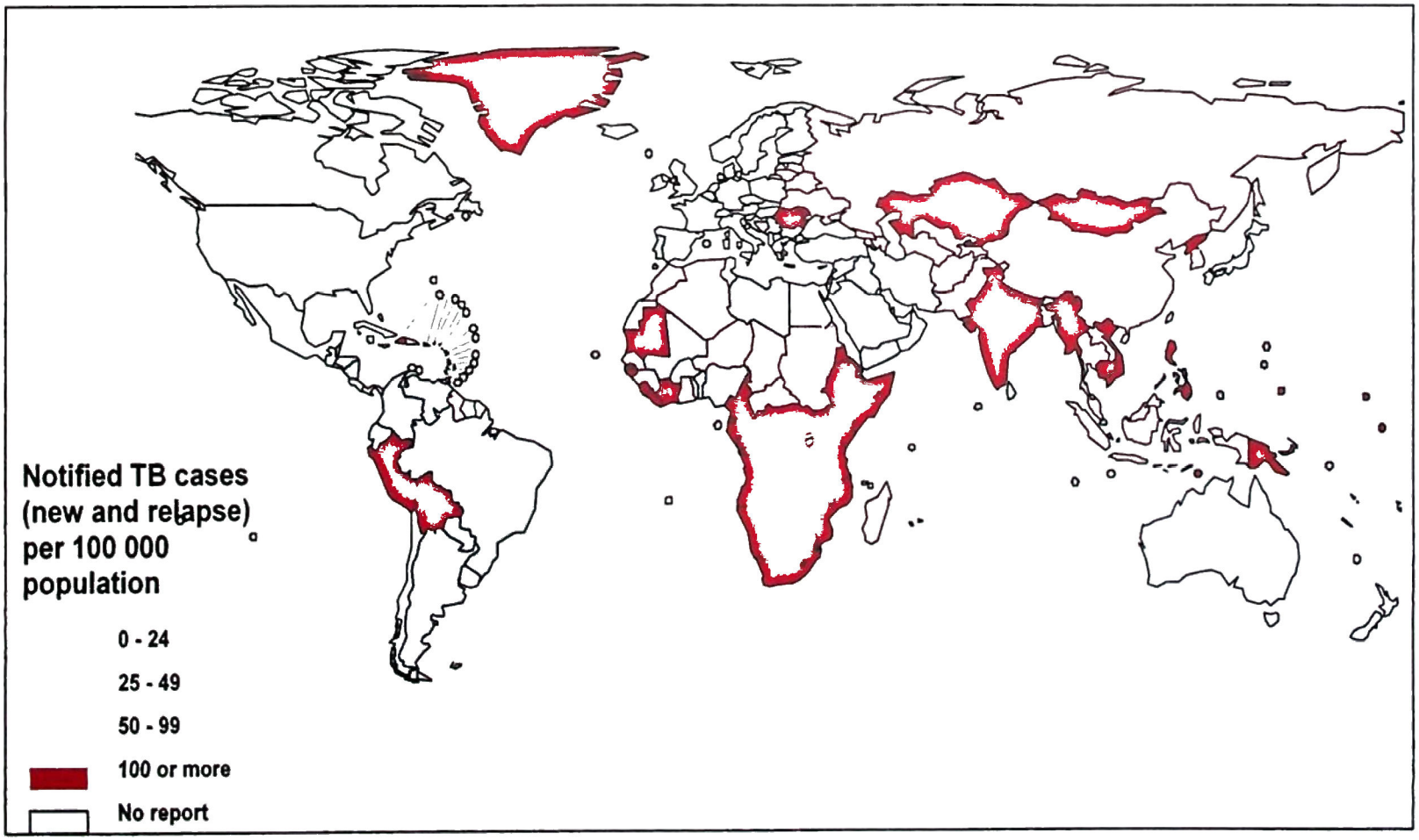

Figure 1.1: Tuberculosis notification rates, 2004. (Adapted from WHO report 2006)

1.3 *Mycobacterium tuberculosis* infection

M. tuberculosis belongs to the Mycobacteriaceae family and Actinomycetales order. Humans are the only reservoirs. *M. tuberculosis* is an aerobic, non-spore forming, non-motile, slightly curved or straight rod bacterium of 0.2 - 0.6 X 1.0 - 10 μm in length. The cell wall of *M. tuberculosis* contains high content of complex lipids. One of the components is mycolyl-arabinogalactan which acts as a hydrophobic permeability barrier that prevents penetration of common aniline dyes.

TB is spread through the air from one person to another. Primary infection begins upon inhalation of 1-10 aerosolized bacilli. The bacteria can settle in the lungs and begin to grow. From there, they can move through the blood to other parts of the body, such as the kidney, spine, and brain. This pathogenic mycobacteria can survive in the hostile habitat of the macrophage, the main immune cell that attract the bacilli. Following the infection of *M. tuberculosis*, 30% of individuals will become infected, with about 40% of these individuals develop primary active TB while the remaining 60% develop latent infection (Figure 1.2). Latent infection is described as a clinical syndrome that occurs after an individual has been exposed to *M. tuberculosis*. During that particular stage, the immune response has been generated to control the pathogen and force it into a dormant stage. Individuals with latent TB do not transmit the disease. After years of dormancy, this organism may start to replicate, leading to reactivation of infection and clinical disease. Individual who is latently infected, can develop active disease via either endogenous reactivation of the latent bacilli or exogenous reinfection with a second mycobacterial strain. Approximately, 2 - 23% of immunocompetent patients with latent TB will reactivate at a later date, while patients with HIV develop reactivation of TB at a rate of 5 - 10% per year (Figure 1.2) due to progressive depletion and dysfunction of the macrophage (Goletti *et al.*, 1996).

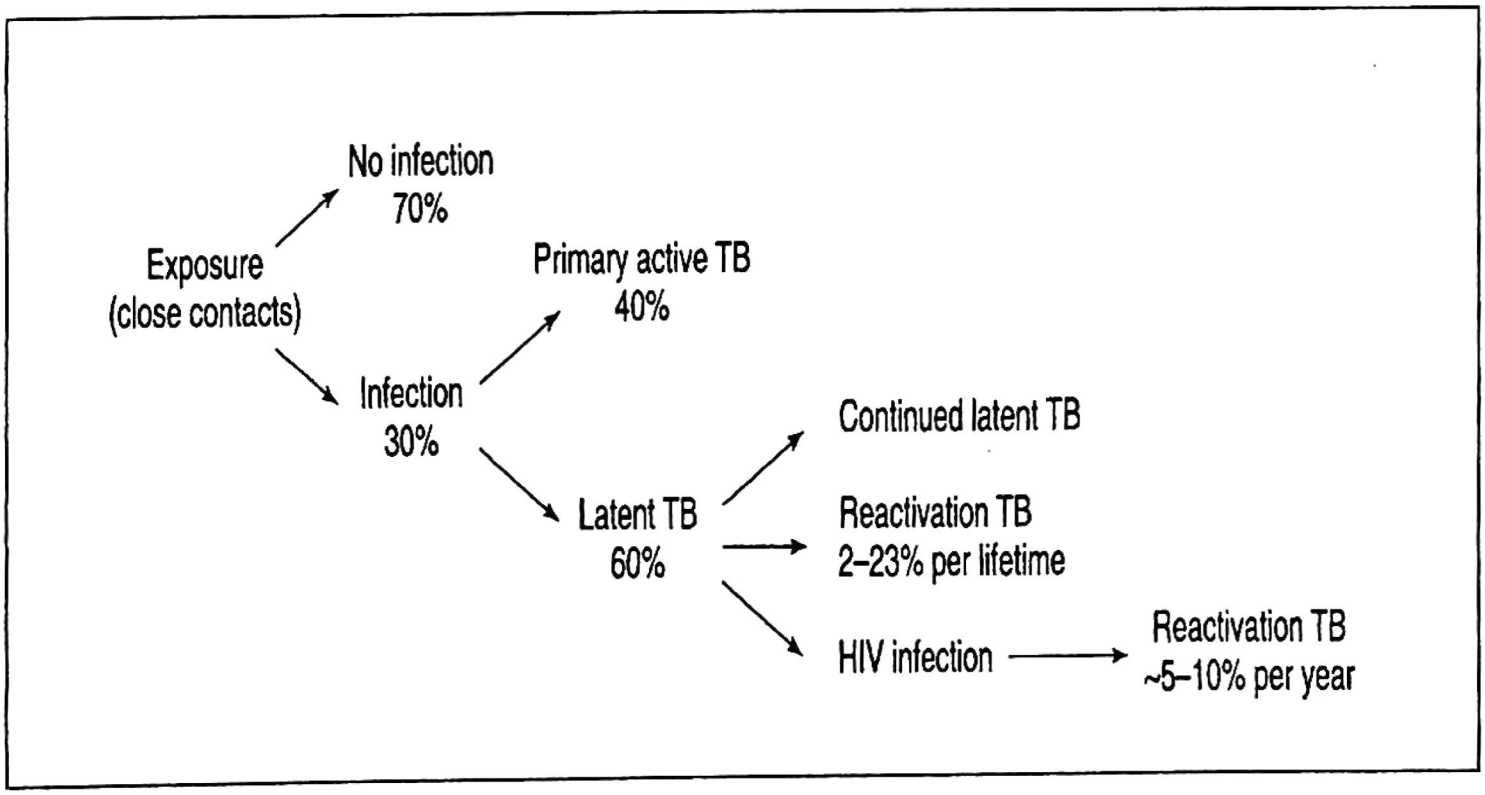

Figure 1.2: Outcomes associated with exposure to *M. tuberculosis*. (Adapted from Parrish *et al.*, 1998).

The capacity to limit the proliferation of tubercle bacilli within macrophage resides largely with $CD4^+$ T-helper (Th) lymphocytes. Despite HIV patients, the reactivation of the pathogen will most probably occur in people with immunosuppression due to age, corticosteroids and malnutrition (Flynn, 2004).

The pathogenicity of the organism is determined by its ability to escape the host immune response as well as eliciting delayed type hypersensitivity (DTH). DTH is used as a general category to describe all those hypersensitivity reactions that take more than 12 hours to develop, which involve cell-mediated immune (CMI) reactions rather than humoral immune reactions. DTH skin testing or Mantoux reaction is carried out to determine previous exposure to TB by injection of tuberculin into the skin of an individual in whom previous infection with the mycobacterium had induced a state of CMI. The reaction is characterized by erythema and induration which appears only after several hours and reaches a maximum at 24 - 48 hours.

1.4 Diagnosis

The most common method used to diagnose TB is by smear microscopy or known as Acid-fast bacillus (AFB) shown in Figure 1.3 which is the most popular, rapid and inexpensive method. However, the reliability of this method is highly dependent on the experience of the laboratory personnel and on the number of organisms present in the specimen. Another method known as the current 'gold standard' is by culture whether on solid or liquid media.

One of the latest technologies used to diagnose TB is by nucleic acid amplification (NAA)-based assays. NAA refers to a technique in which the nucleic acid (DNA or RNA) of an

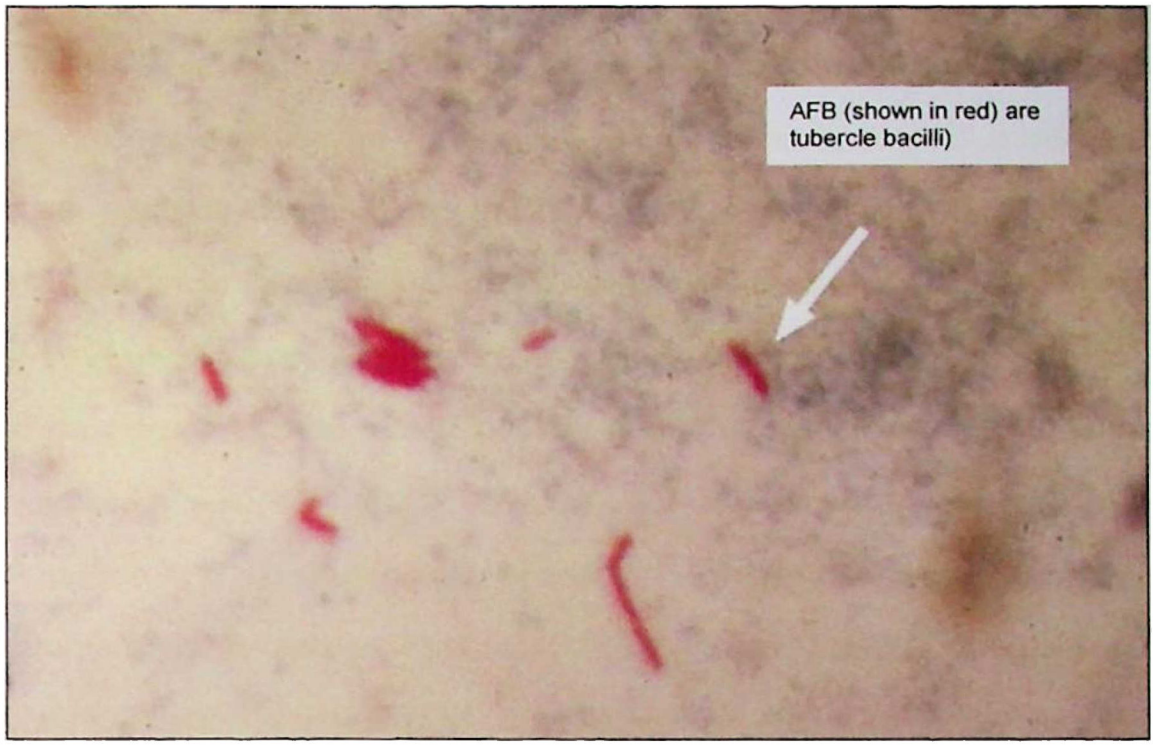

Figure 1.3: AFB smear

organism is amplified by as much as 40 orders of magnitude, after which a probe detects a target sequence of DNA or RNA unique to a particular organism. Compared to smear and culture technique, sensitivity and specificity of NAA are usually very high (Pfyffer, 1999) and can detect as few as 10 organisms in 1 ml of clinical sample (Schluger & Rom, 1995). NAA method can also reduce the diagnostic time from weeks to days. Currently, two NAA methods are available commercially, the Enhanced *Mycobacterium tuberculosis* Direct Test (Gen-Probe®) and the Amplicor® *Mycobacterium tuberculosis* Test (Roche Diagnostic Systems) (reviewed by Soini & Musser, 2001). Both products have been approved by the Food and Drug Administration USA (FDA) in 1999 for direct detection of *M. tuberculosis* from clinical specimens (CDC, 2000). The NAA test can enhance diagnostic speed, but could not replace AFB smear or culture because the test cannot distinguish between live and dead organisms. In addition, NAA test require complex equipment as well as highly technical staff. Therefore, clinicians should interpret the NAA test results based on the clinical situation and the test should be performed at the request of the clinician (Soini & Musser, 2001).

Besides the AFB, culture and NAA methods of TB diagnosis, susceptibility testing is one of the available alternatives if the culture remains positive over a longer period of time. Drug susceptibility testing is mandatory on initial isolates of *M. tuberculosis* and related species from all patients. Susceptibility testing is conducted to monitor a possible development of drug resistance. Conventional method for drug susceptibility is by testing on solid media (Middlebrook 7H11 or LJ). Another recent method of drug susceptibility testing is by radiometric liquid culture system (BACTEC) which provides a vial containing a substance [para-nitro-alpha-acatylamine-hydroxypropiophenone (NAP)], which selectively suppress the growth of *M. tuberculosis* complex species. Among members of the *M. tuberculosis* complex are *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microtii*. Each member of the

M. tuberculosis complex is pathogenic. If a subculture from the initial vial fails to demonstrate growth in the NAP, that is presumptive evidence for a species within *M. tuberculosis* complex.

1.5 Symptoms and treatments

The symptoms of TB depend on the site where the bacteria are growing whether in pulmonary or extrapulmonary. In the lungs, symptoms such as coughing for 3 weeks or longer, pain in the chest and coughing out blood or sputum are very common. Only active TB patients will show some other possible symptoms which are; weakness or fatigue, weight loss, fever, sweating at night and reduced appetite. Besides pulmonary TB, most extrapulmonary forms of TB includes; TB meningitis, tuberculous lymphadenitis, pericardial TB, pleural TB and disseminated or miliary TB. People with HIV, infants and young children seem to have an increased risk for extrapulmonary TB.

Containment of TB has been carried out by the WHO-recommended "directly observed treatment short course" (DOTS) strategy. This treatment involves TB patients observed taking every single dose drug for the first 2 month of the 6 to 8 month treatment regimens. More than 17 million patients benefited from the DOTS strategy, but in some cases multi-drug resistant TB (MDR-TB) occurs when the treatment is incomplete (Girard *et al.*, 2005). MDR-TB is defined as strains of *M. tuberculosis* resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. The first documented case of MDR-TB was in a lung transplant patient in 1999 (Lee *et al.*, 2003). Transplant patients are chronically immunosuppressed and in that study, the donated lungs were from a recent Chinese immigrant who was at high-risk for previous exposure. Fortunately, fluctuations and variations of isoniazid, rifampicin, pyrazinamide and rifabutin were successful in saving the patient.

1.6 Immune response against TB

Immune response involved in TB infection is complex. The components involved are T cells ($CD4^+$ and $CD8^+$), cytokines (IFN-γ, TNF-α, IL-12 and IL-6) and macrophages (Flynn, 2004). The immune response may also differ in acute and chronic infection. Four stages of pulmonary TB (Figure 1.4) have been reviewed by van Crevel *et al.* (2002). The first stage is the inhalation of tubercle bacilli. After an incubation period of 4 to 12 weeks, alveolar macrophage will ingest the bacilli and destroy them. These depend on the intrinsic microbicidal capacity of host phagocytes as well as the virulence factors of the ingested mycobacteria.

Mycobacteria which escape the first stage will enter the second stage where three scenarios could occur. The first scenario is when the host failed to contain the pathogen and die. Secondly, the mycobacteria may spread throughout the body when the host immune response is weak (normally occurs in immunocompromised patients) causing active disease. The third scenario is when the host immune response and the virulence of *M. tuberculosis* are balanced and the intracellular bacteria are contained within the macrophage. Macrophage disruption will attract blood monocytes and other inflammatory cells to the lungs. Monocytes will differentiate into macrophages and ingest the mycobacteria but will not destroy them. Little tissue damage occurs at this stage. T cell immunity will develop after 2 to 3 weeks of infection, leading to proliferation of antigen specific T lymphocytes within the early lesions. Host immune system isolates the primary site of infection by granuloma formation. The granuloma contains lymphocytes including $CD4^+$ and $CD8^+$ T cells as well as B cells. In addition, fibroblasts and other cells can be present within the granuloma (Co *et al.*, 2004). The granuloma functions to limit the spread

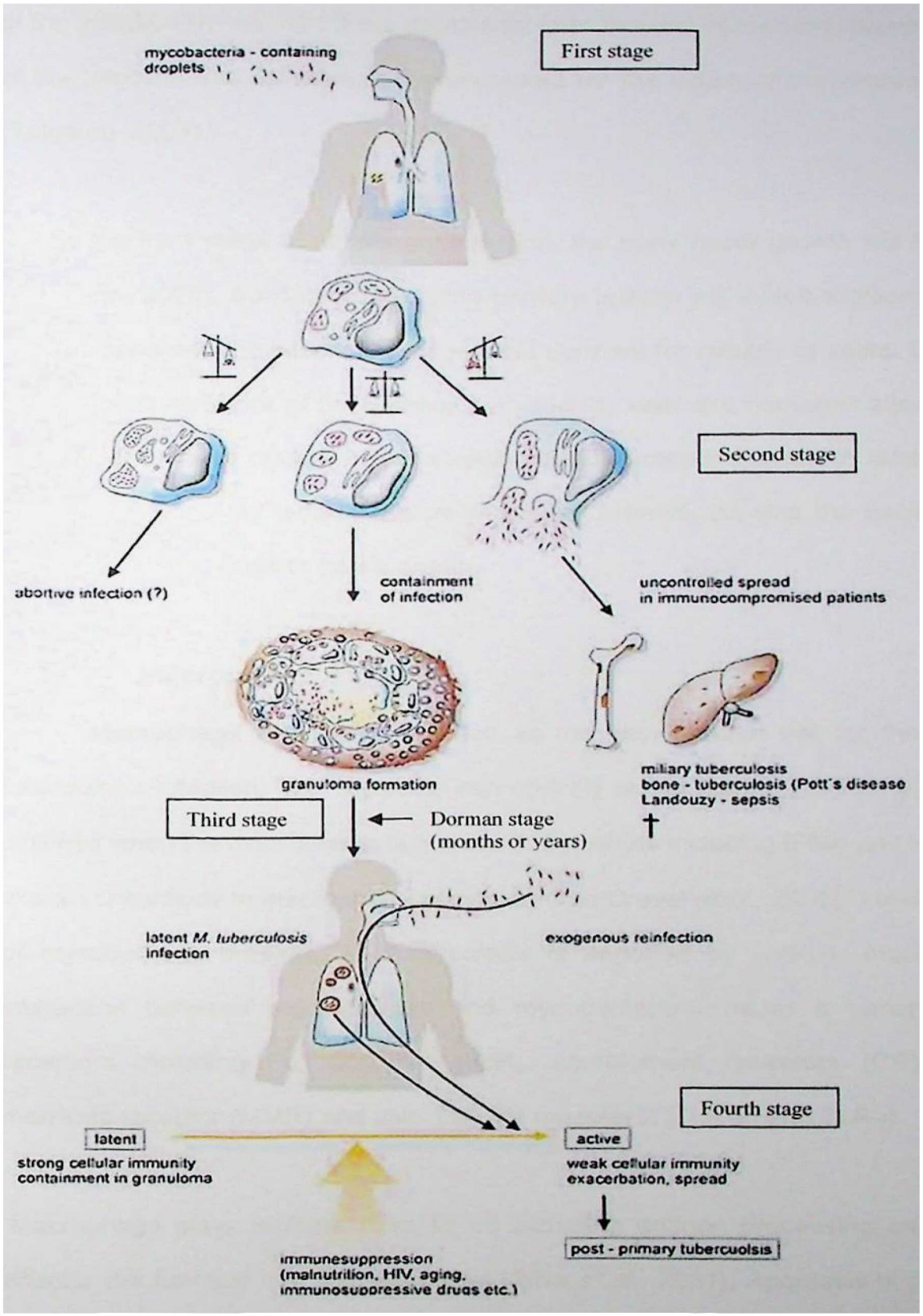

Figure 1.4: Four stages of pulmonary TB (Modified from Kaufmann & Ulrichs, 2003)

of the infection by walling off the organisms from the rest of the lung, prevents metastasis of the infection and providing an environment for the action of the immune components (Salgame, 2005).

During the third stage of pulmonary infection, the early bacilli growth will stop (Ulrichs & Kaufmann, 2003). Solid necrosis in the primary lesions will inhibit extracellular growth of mycobacteria and the infection may become dormant for months or years. During the final stage, any disturbance of the balance between the host and pathogen after weakening of the cellular immune response causes endogenous exacerbation which leads to active TB. Cavity formation may lead to rupture of nearby bronchi, causing the bacilli to spread to other parts of the lungs or host's organ.

1.6.1 Macrophage

Macrophage has been identified as the key immune cell for the control of *M. tuberculosis* infection. The organism can multiply within resting macrophage but become inhibited when the macrophage is activated. Cytokines including IFN-γ and TNF-α and also vitamin D involves in macrophage activation (van Crevel *et al.*, 2002). Following inhalation of mycobacteria droplets, *M. tuberculosis* is engulfed by alveolar macrophages. The interaction between macrophages and mycobacteria involves a variety of host cell receptors including Fc receptors (FcR), complement receptors (CR), macrophage mannose receptor (MMR) and also Toll-like receptor 2 (TLR-2) and TLR-4.

Macrophage plays multiple roles in TB including antigen processing and presentation, effector cell function and also apoptosis (Silva *et al.*, 2001). Apoptosis of phagocytic cells may prevent dissemination of infection and reduces viability of intracellular mycobacteria. Klinger and colleagues (1997) have demonstrated that apoptosis associated with TB is

mediated through a downregulation of bcl-2, an inhibitor of programmed cell death. The activated macrophage produces reactive oxygen intermediates (ROIs) by oxidative burst and reactive nitrogen intermediates (RNIs) via inducible nitric oxide synthase (iNOS2). Cooper *et al.* (2000) provided evidence that ROI-mediated control is important during early infection by the observation of a 10-fold higher bacterial numbers in the lungs of p47phox knockout (KO) mice, compared to wild-type controls, after aerosol challenge with *M. tuberculosis*. The p47phox is a phagosome oxidase component critical for the activity or assembly of the functional oxidase. RNIs are the critical effector molecules against *M. tuberculosis* in the mouse. Moreover, mice deficient in NOS2 activity are very susceptible to acute or chronic *M. tuberculosis* infection compared to wild-type mice (MacMicking *et al.*, 1997; Scanga *et al.*, 2001).

Macrophage activation also involves natural-resistance-associated macrophage protein (*Nramp1*) gene and vitamin D. *Nramp1* is an interesting gene involved in macrophage activation and mycobacterial killing (Blackwell *et al.*, 2000). The protein is an integral membrane protein which belongs to a family of metal ion transporters. These metal ions, particularly Fe^{2+}, are involved in macrophage activation and generation of toxic antimicrobial radicals (Zwilling *et al.*, 1999). Following phagocytosis, *Nramp1* becomes part of the phagosome. *Nramp1* mutant mice display reduced phagosomal maturation and acidification (Hackam *et al.*, 1998).

Macrophage suppresses the growth of *M. tuberculosis* by the helps of active metabolite of vitamin D, 1, 25-dihydroxyvitamin D (Rockett *et al.*, 1998). A recent study among Gujarati Hindus, a mainly vegetarian immigrant population in London, showed that vitamin D deficiency was a risk factor for TB (Wilkinson *et al.*, 2000). Eventhough activated macrophage can sometimes kill virulent *M. tuberculosis* (Sato *et al.*, 1998) but it is

generally cannot eliminate the infection entirely. Therefore, other components of the immune system including cellular and humoral immune responses participate to eliminate the mycobacteria.

1.6.2 Cellular Immune Response

Van Crevel *et al.* (2002) has discussed three processes that contribute to the initiation of cellular immune response against TB; antigen presentation, costimulation and cytokine production. Antigen presentation involves $CD4^+$ T cells, $CD8^+$ T cells and unconventional T cells including CD1 and γδ T cells. In general, $CD4^+$ T cells help to amplify the host immune response by activating effector cells and recruiting additional immune cells to the site of disease, whereas $CD8^+$ T cells are important during the latent stage of TB infection, which act as cytotoxic T cells (CTL) by lysing infected cells (Schluger & Rom, 1998) through production of various cytokines such as IFN-γ and TNF-α. Within a week of infection with virulent *M. tuberculosis*, the number of activated $CD4^+$ and $CD8^+$ T cells in the lung-draining lymph nodes increases (Feng *et al.*, 1999; Serbina *et al.*, 2000).

Basically, $CD4^+$ Th lymphocytes differentiate from precursor Th0 cells under the control of cytokines such as IL-2 and IL-4 into two functionally distinct subsets either type 1 (Th1) or type 2 (Th2) cells (Figure 1.5). Th1 secretes cytokines such as IL-2, IFN-γ, TNF-α and IL-12 resulting in macrophage activation and induction of CMI. In contrast, Th2 secretes IL-4, IL-5, IL-6 and IL-10 resulting in the induction of humoral immunity by antibody production. *M. tuberculosis* resides primarily in a vacuole within the macrophage resulting in major histocompatibility complex (MHC) Class II presentation of mycobacterial antigens to $CD4^+$ T cells. The HIV epidemic has demonstrated that the loss of $CD4^+$ T cells greatly increases susceptibility of the host to both acute and reactivation TB (reviewed by Flynn, 2004).

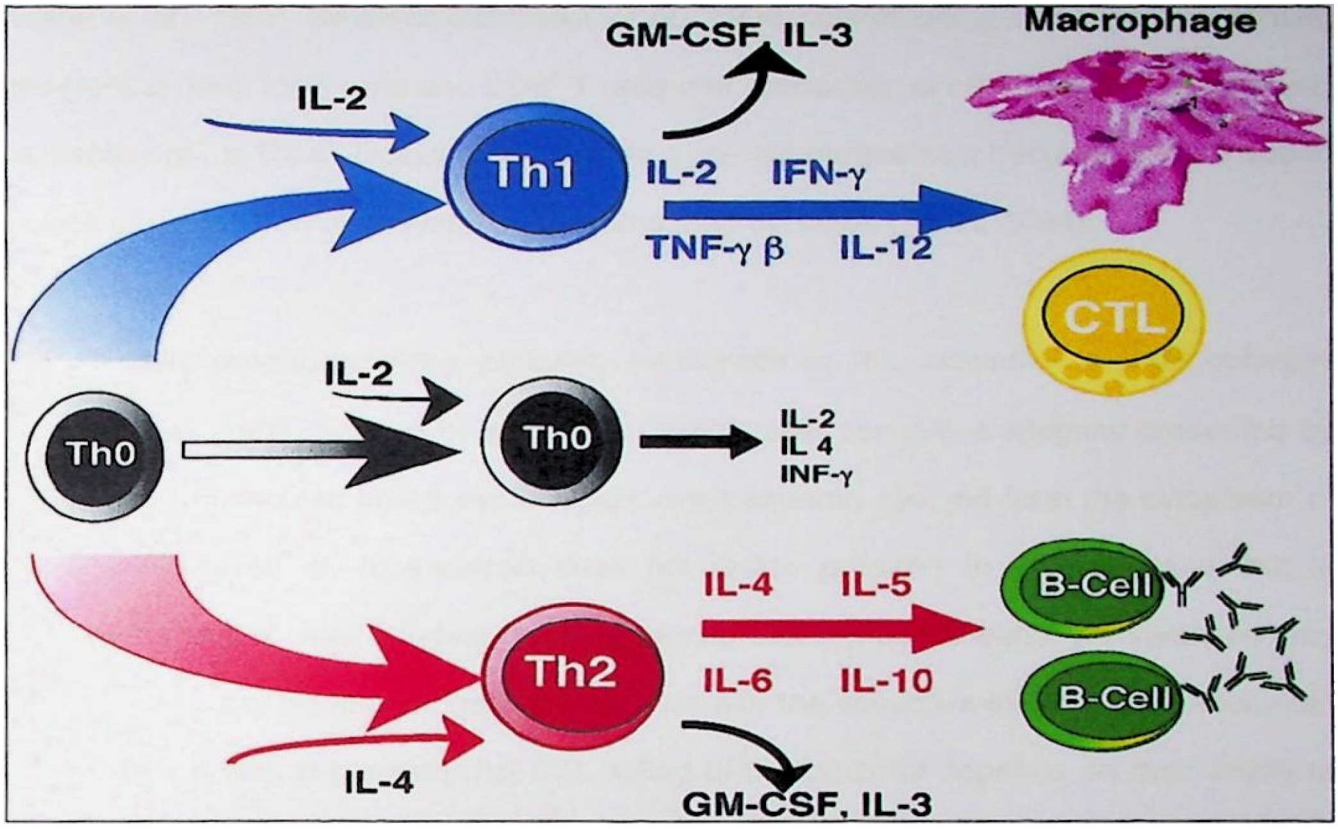

Figure 1.5: $CD4^+$ T helper lymphocyte subsets upon activation. (Adapted from Cohen *et al.*, 1998).

The other possible roles of $CD4^+$ T cells in controlling TB infection include, apoptosis (Keane *et al.*, 1997; Balcwicz-Sablinska *et al.*, 1998), conditioning of antigen presenting cells (APCs), help for B cells and $CD8^+$ T cells and production of other cytokines. However, the inability of the $CD4^+$ T cells to completely eliminate intracellular bacteria may be due to the lack of recognition or activation of infected macrophages (Flynn, 2004).

$CD8^+$ T cells producing IFN-γ probably participate in the activation of macrophages (Caruso *et. al.*, 1999; Scanga *et al.*, 2000). $CD8^+$ T cells recognize antigens presented by MHC Class I molecules and these antigens are frequently derived from the cytoplasm of the cells. However, *M. tuberculosis* does not reside primarily in the cytoplasm but in vacuoles inside the cells. Studies have suggested that the bacilli within the vacuoles may have access to the cytoplasm, perhaps via a pore in the vacuole's membrane (Teitelbaum *et al.*, 1999). It was suggested that CTL killing of the bacteria depends on their ability to deliver potent bactericidal proteins such as granulysin from their granules (Silva *et al.*, 2001). Lysis of target cells by $CD8^+$ T cells can occur via perforin and granzymes or the Fas/FasL (CD95L) pathway resulting in apoptotic cell death or release of bacteria from an infected cell into the granuloma (Canaday *et al.*, 2001). The importance of $CD8^+$ T cells in TB was reported by Behar *et al.* (1999), when β2-microglobulin (β2-m) and transporter associated protein (TAP1) KO mice, which cannot generate $CD8^+$ T cells, were infected with *M. tuberculosis* and resulted in an exacerbated course of infection.

As mentioned earlier, unconventional T cells such as CD1 and γδ T cells also play a role in host defense against mycobacterial infection. Both cells produce type 1 cytokines, most importantly IFN-γ which activates anti-mycobacterial activities in macrophages (Raupach & Kaufmann, 2001). CD1-restricted αβ T-lymphocytes are thought to be activated by

mycobacterial lipids (Agger & Andersen, 2002). The CD1 family consists of antigen-presenting molecules encoded by genes located outside of the MHC. CD1 genes are conserved among mammalian species and are expressed on the surface of the cells involved in antigen presentation, notably dendritic cells (DCs). The CD1 system is involved in activation of CMI response against mycobacterial infection. It is the least common T cell subset in human peripheral blood and lung. In humans, most of these T cells express neither CD4 nor CD8 and are referred to as double-negative (DN) cells. In mice, CD1d-restricted natural killer (NK) T cells are activated by mycobacterial cell wall components and are involved in early granuloma formation (Apostolou *et al.,* 1999).

Meanwhile, γδ T cells are large granular lymphocytes, non-MHC restricted that can develop a dendritic morphology in lymphoid tissues and function as CTL. Unconventional γδ T cells are activated by small phosphorylated metabolites (Agger & Andersen, 2002). It was suggested that γδ T cells may play a role in early immune response against TB and is an important part of the protective immunity in patients with latent infection (reviewed by Raja, 2004).

The second process that leads to the initiation of cellular immunity is by costimulation. Antigen presentation only leads to T cell stimulation in the presence of several costimulatory signals. The most well known costimulatory signals for T cell stimulation are B-7.1 (CD80) and B-7.2 (CD86). These molecules are expressed on macrophages and DCs and bind to CD28 and to CTLA-4 on T cells. In the absence of proper costimulatory signals, antigen presentation may lead to an increased apoptosis of T cells (Hirsch *et al.,* 1999 & 2001).

Finally, the production of cytokines may also contribute to the initiation of cellular immunity in TB infection. Several cytokines produced by activated macrophages and DCs are essential for stimulation of T lymphocytes. These include IFN-γ, TNF-α, IL-4, IL-12, IL-18 and IL-15. IFN-γ is produced by T cells from healthy purified protein derivative positive (PPD+) subjects as well as those with active TB. IFN-γ is important to activate macrophage as well as TNF-α that synergize with IFN-γ to induce antimycobacterial effects. Individuals lacking receptors for IFN-γ suffer from recurrent, sometimes lethal mycobacterial infections (Holland *et al.*, 1998). There are three possible cells responsible for nonspecific production of IFN-γ as reviewed by van Crevel *et al.* (2002). First, before adaptive T cell immunity has fully developed, NK cells may be the main producer of IFN-γ, either in response to IL-12 and IL-18 (Iho *et al.*, 1999) or directly by exposure to mycobacterial oligodeoxynucleotides (Garcia *et al.*, 1999). Second, lung macrophages were found to produce IFN-γ in *M. tuberculosis*-infected mice (Wang *et al.*, 1999). Third, the γδ T cells and CD1-restricted T cells may produce IFN-γ during early infection.

Besides IFN-γ, stimulation of monocytes, macrophages and DCs (Henderson *et al.*, 1997) with mycobacteria or mycobacterial products induce the production of TNF-α. TNF-α plays a role in granuloma formation, induces macrophage activation and has immunoregulatory properties (Orme & Cooper, 1999; Tsenova *et al.*, 1999). In addition to TNF-α, IL-12 has a crucial role in the induction of IFN-γ production (O'Neill & Greene, 1998). IL-12 is produced mainly by phagocytic cells. In TB, IL-12 has been detected in lung infiltrates, in pleurisy, in granulomas and in lymphadenitis (reviewed by van Crevel *et al.*, 2002). The expression of IL-12 receptors is also increased at the site of disease (Zhang *et al.*, 1999). Together with IL-12, IL-18 and IL-15 seem to be important in the IFN-γ axis (O'Neill & Greene, 1998). IL-18 KO mice was found to be highly susceptible to *M. tuberculosis* (Sugawara *et al.*, 1999)

and in mice infected with *M. leprae*, resistance is correlated with a higher expression of IL-18. Moreover, *M. tuberculosis*-mediated production of IL-18 by peripheral blood mononuclear cells (PBMC) is reduced in TB patients and this reduction may be responsible for reduced IFN-γ production (Vankayalapati *et al.*, 2000).

Another cytokine that have been studied regarding TB infection is IL-4. Inhibition of IL-4 production did not seem to promote cellular immunity. IL-4-/- mice displayed normal instead of increased susceptibility to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of TB development (Erb *et al.*, 1998; North, 1998).

1.6.3 Humoral Immune Response

Researchers argued about the role of antibodies in host defense against *M. tuberculosis* which was believed that intracellular pathogens cannot be reached by antibodies. However, intracellular pathogens are found in the extracellular space prior to their entry into cells. Furthermore, certain antibodies can enter or mediate biological effects inside cells (reviewed by Glatman-Freedman & Casadevall, 1998). Previous work demonstrated protective effects of antibodies against infection with the intracellular fungus *Cryptococcus neoformans*, as well as other intracellular pathogens (reviewed by Casadevall, 1998). Recently, there are several studies that support the role of antibodies in *M. tuberculosis* infection (Bosio *et al.*, 2000).

M. tuberculosis will infect pulmonary lymph nodes and other organs such as spleen and liver. The dissemination of *M. tuberculosis* probably occurs via entry into alveolar macrophages and via interaction with epithelial cells (Pethe *et al.*, 2001). Heparin binding hemagglutinin adhesion (HBHA) is a surface-exposed glycoprotein involved in the binding

of *M. tuberculosis* to epithelial cells. Administration of *M. bovis* coated with anti-HBHA monoclonal antibodies (mAbs) intranasally reduced colony forming unit (CFU) in the spleen (Pethe *et al.,* 2001) suggesting that anti-HBHA antibodies interfered with mycobacterial dissemination.

Despite playing a role in mycobacterial dissemination, humoral immune response also involved in cytokine expression. A study on the effect of antibodies to PPD on TNF-α expression by monocytes was carried out by Hussain *et al.* (2000). TNF-α is a pro-inflammatory cytokine involved in host response against *M. tuberculosis*, as well as the immunopathology of TB (Engele *et al.,* 2002). TNF-α secretion by PPD-stimulated monocytes from PPD skin test-negative donors was enhanced in the presence of heat-inactivated plasma obtained from patients with pulmonary TB (Hussain *et al.,* 2000). Furthermore, TNF-α secretion directly correlated with plasma concentration of IgG1 to PPD and adsorption of IgG1 from plasma samples led to reduction of TNF-α secretion suggesting a potential role for certain antibody in mediating a biological effect via cytokine release.

Mycobacteria enter the host via the mucosa, therefore antibodies in the secretions could play a role in host defense against mycobacteria. In another study, high titers of antibodies against mycobacteria were observed in mice administered with human gammaglobulin formation and challenged after 2 hours by the intranasal route (Acosta *et al.,* 2003). In addition, mice lungs showed significantly decreased CFU 24 hours after the challenge.

1.7 BCG – the current vaccine

The bacille Calmette-Guerin (BCG) vaccine was named after the French scientists Calmette and Guerin in 1908. They isolated *M. bovis* from a cow with tuberculous mastitis (a common symptom in cattle with bovine TB). They cultured this isolate in a glycerinated, beef/bile/potato medium, subculturing every three weeks for a total of 231 passages, over a period of 13 years. The so-called BCG Pasteur strain was widely distributed throughout the world. As a result, many daughter substrains developed and numerous changes, gene deletions as well as continued passage have attenuated BCG to almost complete avirulence (Behr *et al.*, 1999a; Sabino *et al.*, 2004). A review by Brosch *et al.* (2001), found that there are at least 18 variable regions of difference (RD), representing 120 genes present in *M. tuberculosis* H37Rv but absent in BCG Pasteur. One region, RD1, is missing in all BCG strains but is present in all *M. bovis* and *M. tuberculosis* strain (Mahairas, 1996; Cole *et al.*, 1998; Behr *et al.*, 1999b; Brosch, 2000). This RD1 region might contain some of the genes involved in virulence or regulators of virulence genes. These might account for the phenotypic differences between the vaccine strain and *M. tuberculosis.*

BCG was first tested in infants in 1921 as an oral vaccine. New methods of administration were introduced later, such as intradermal, multiple puncture and scarification. The most widely used BCG vaccine substrains include Connaught, Danish, Glaxo, Moreau, Pasteur and Tokyo (Oettinger *et al.*, 1999). In 1974, BCG vaccination has been included in the WHO Expanded Program on Immunization, resulting in more than three billion doses injected worldwide and approximately 100 million immunizations in children each year. BCG is routinely administered to newborns in countries where TB is endemic and in some lower-incidence countries. A common minor side effect of BCG immunization is the production of induration and ulceration at the vaccination site.

1.7.1 Efficacy and effectiveness of BCG

BCG vaccination does not prevent infection with *M. tuberculosis* but helps the host to retard the growth of organisms at the primary site of infection (Smith & Starke, 2002). Although BCG can protect against childhood TB, particularly meningitis, it cannot protect against adult TB. Furthermore, in immunocompromised patients, BCG may cause disease (Cunningham *et al*, 2000).

There is minimal evidence that BCG-induced protection lasts longer than 15 years (Fine, 2001). Several clinical trials carried out to study the effect of revaccination with BCG during adulthood revealed that BCG does not prevent against adult pulmonary disease (Tala-Heikkila *et al*., 1998; Leung *et al*., 2001). The lack of efficacy following BCG vaccination was not only observed in humans but also in animals (reviewed by Hewinson, 2005). BCG vaccination of calves within hours of birth is highly effective at protecting animals against bovine TB, but revaccination of these animals at six weeks is no longer effective (Buddle *et al*., 2003). In mouse models, revaccination with BCG does not stimulate BCG immunity in the lung, although protective responses in the spleen were seen (Derrick *et al*., 2004). Efficacies of BCG have varied widely. Several controlled clinical trials to determine its efficacy in preventing pulmonary TB has ranged from zero protection in the southern United States and in Chingleput, South India, to approximately 80% in the United Kingdom (UK) (Datta *et al.,* 1999; Orme, 1999; Wang & Xing, 2002; Clements, 2003).

There are some possible factors that result in the variable responses in the BCG field trials. First is the variation in vaccine strains administered. There is therefore a lack of comparability between different vaccination studies due to the current use of six different BCG substrains (Dietrich *et al*., 2002). The second factor is the presence of environmental mycobacteria. A wide range of environmental mycobacterial spesies such as *M. avium* and

M. kansasii are found in soil and water (Kamala *et al.,* 1998). Repeated exposure to these environmental mycobacteria can sensitize individuals and stimulate CMI responses that can be measured both *in vitro* (Black *et al.,* 2001a & 2002) and in the skin test (Black *et al.,* 2001b; Weir *et al.,* 2003). The third factor is the host where the mycobacteria reside. Variation in clinical trials conducted in different populations has led to the hypothesis that genetic or nutritional factors might influence the immune responses. Finally, the failure of BCG may be due to the absence of important T cell antigens in BCG which are needed to stimulate effector T cells against *M. tuberculosis.* (Norazmi *et al.,* 2005).

1.7.2 Advantages of BCG

BCG still has many advantages eventhough its role in affording protection is controversial. There are two areas in which BCG vaccine has shown consistent benefits (Smith & Starke, 2002). First, BCG confers protection against disseminated TB disease with high levels of protection against miliary TB and tuberculous meningitis especially among vaccinated infants. Secondly, BCG protects against leprosy (reviewed by Clements, 2003). BCG is the most widely used vaccine worldwide and is the safest vaccine identified so far. BCG also elicits Th1 mediated immune response and can mimic the natural infection without causing disease. Furthermore, BCG is inexpensive and easy to produce. Being a live vaccine, BCG has become a potential novel vaccine vehicle that can continuously express recombinant foreign antigens (Ohara & Yamada, 2001) whether carried in BCG either on episomally replicating vectors or alternatively through integration into the BCG chromosome.

1.8 Candidate antigens of *M. tuberculosis*

1.8.1 Mtb8.4

Mtb8.4 was identified through biochemical purification of immunodominant T cell antigens from TB culture filtrates. Mtb8.4 is capable of eliciting proliferation and IFN-γ production in PBMCs from PPD+ donors (Evans *et al.*, 2004). Preliminary studies in mice have shown that immunization with Mtb8.4-DNA can provide partial protection from TB challenge (Coler *et al.*, 1998 & 2001). In addition, Mtb8.4 protein-microsphere formulation developed by Evans *et al* (2004) has the capability of eliciting strong CMI and humoral immune responses against Mtb8.4 following a single immunization. This Mtb8.4 protein-microsphere formulation elicits $CD8^+$ T cell with cytolytic activity equivalent to intramuscular (i.m) plasmid DNA immunization and antibody responses that are stronger than monophosphoryl lipid A (MPL®) adjuvant.

1.8.2 Ag85B

Antigen 85 (Ag85) complexes is a protein of the BCG cell wall. It is one of the major secreted proteins in *M. tuberculosis* or BCG culture filtrates. It consists of three structurally related components, Ag85A, Ag85B and Ag85C, with molecular weights of 29-30 kDa (reviewed by Groves, 1997). These antigens are highly conserved among all mycobacterial species. Their function is to bind to fibronectin and thus may be involved in the macrophage phagocytic process. Immunization of mice with Ag85 complex induced effective protective immunity against multiplication of *M. leprae* in the foot pads of mice (Naito *et al.*, 2000).

In 1999, Lee & Horwitz identified the immunodominant epitopes of the 30, 32 and 16 kDa major extracellular proteins of *M. tuberculosis.* Outbred guinea pig was immunized with

each of the three proteins. Proliferative response assay showed that the 30 kDa is the most immunogenic of the three proteins tested. The 30 kDa which is also known as Ag85B consists of 285 amino acids (Harth *et al.*, 1997) and contains multiple human T cell epitopes that are scattered throughout the entire protein. Several of these are important T cell epitopes that could stimulate T cells by Human Leukocyte Antigen-DR (HLA-DR)-mismatched APCs (Mustafa *et al.*, 2000). HLA-DR is a glycosylated cell surface transmembrane protein expressed on APCs and constitutively expressed on monocytes. Expression of HLA-DR by monocytes is essential for the presentation of peptides derived from microbes to $CD4^+$ T cells to initiate a specific immune response (Cheadle, 1993).

Immunoreactive epitopes of the 30 kDa on the basis of mapping in guinea pig frequently overlapped with immunoreactive epitopes of the *M. bovis* BCG 30 kDa protein identified in the basis of mapping in healthy PPD+ and BCG-vaccinated individuals (Roche *et al.*, 1994; Silver *et al.*, 1995). Moreover, three of these seven immunoreactive regions of guinea pig and humans (amino acid 101 to 115, 126 to 140 and 261 to 275) overlap with T cell epitopes of the 30 kDa protein predicted by a newly developed computer program (EpiMer) that predicts putative T cell epitopes by searching for the HLA-binding motifs on a given protein sequence (Meister *et al.*, 1995).

1.9 Experimental vaccines developed against TB

Many approaches have been used by different researchers in order to develop a better vaccine against TB. Among the approaches include, recombinant BCG (rBCG), DNA vaccination, subunit vaccine, auxotroph vaccine and live vaccine. However, this chapter will only focus on the DNA vaccine and rBCG approaches.