View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repository@USM

CLONING, EXPRESSION AND IMMUNOGENICITY OF RECOMBINANT BACILLE CALMETTE-GUERIN (BCG) CONTAINING T AND B CELL EPITOPES OF *Mycobacterium tuberculosis*

ROHIMAH BINTI MOHAMUD

UNIVERSITI SAINS MALAYSIA

2008

CLONING, EXPRESSION AND IMMUNOGENICITY OF RECOMBINANT BACILLE CALMETTE-GUERIN (BCG) CONTAINING T AND B CELL EPITOPES OF *Mycobacterium tuberculosis*

by

ROHIMAH BINTI MOHAMUD

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

UNIVERSITI SAINS MALAYSIA

2008

DEDICATIONS

This thesis is specially dedicated to:

My beloved husband, Mohd. Shamsul Hisham bin Mat Hassan Latfi
My daughters, Fatin Shahirah, Fatin Shakirah and Alissa Shafiyah
My parents, Mohamud bin Awg. Kechik and Siti Bidah binti Loman

Thank you for your love, patience and encouragement.

May Allah bless you all…

ACKNOWLEDGEMENTS

In the name of Allah, the Most Generous and the Most Merciful. All praise are due to Allah for His guidance and peace that give me the ingredients of success.

I wish to acknowledge several people who have been involved directly or indirectly during this research project.

Above all, I would like to thank my supervisor, Prof. Norazmi Mohd. Nor for his support, excellent guidance and supervision throughout the experimental work and also during the writing of this thesis. His guidance and encouragement are greatly appreciated.

I would also like to express my gratitude to my co-supervisor, Dr. Rapeah Suppian for the encouragement, kind guidance and helpful discussions during this study.

I would also like to thank Assoc. Prof. Dr. Nik Soriani Yaacob and Dr. Shaharum Shamsuddin for advice, guidance and overall comments.

I would like to extend my thanks to my friends and colleagues in the laboratory especially, K. Maryam, Asma, Teo, Rafeezul, Zila, Kenny, Boonyin, Syam, K. Ayu, K. La, Fatma, Amir and Wong Vic Cern. Not to forget, my friends in ZFZ and SS group, Eza, Zura, Abdah, K. Su, K. Nurul, Aniek, Yuni, Tini, Bad, Azan, Kak Lin, Venu and Nida. Thank you for both your friendship and assistance during the course of this study.

A special thanks to my parents, Mohamud and Siti Bidah, my brothers and sisters for their support and guidance during this work. Not to forget to my parents-in-law, Hj. Mat Hassan Latfi and Hjh. Rofiah, my brothers- and sister-in-law for their support and encouragement. Finally I wish to acknowledge the greatest support, love and encouragement from my beloved husband, Mohd. Shamsul Hisham for his love and

patience; thanks for being there and encouraging me when needed. My appreciation also goes to my beloved daughters, Fatin Shahirah, Fatin Shakirah and Alissa Shafiyah; they always make me happy.

There are many people that I would like to thank for contributing directly or indirectly to this study. I cannot mention you all so I hope you feel my gratitude.

TABLE OF CONTENTS

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER THREE: RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AFB	Acid fast bacillus
Ag85	Antigen 85
APCs	Antigen presenting cells
B.C	Before Century
BCG	Bacille Calmette Guerin
bp	base pair
CD	Cluster of differentiation
CFU	Colony forming unit
CFP	Culture filtrate protein
CTL	Cytotoxic T lymphocyte
CPM	Count per minute
ddH_2O	Deionised distilled water
DNA	Deoxyribonucleic acid
ETH	Ethambuthol
$\gamma\delta$	Gamma delta
HIV	Human Immunodeficiency Virus
IFN	Interferon
IFN-γR	IFN-γ Receptor
IL	Interleukin
i.m	Intramuscular
INH	Isoniazid
i.p	Intraperitoneal
kDa	Kilo Dalton
KO	Knock out
LTT	Lymphocytes transformation test
MHC	Major histocompatibility complex
MDR-TB	Multi drug resistant-TB
NAA	Nucleic acid amplification
NK	Natural killer
OD	Optical density
ORF	Open reading frame
PAS	P-aminosalicylic acid
PBMC	Peripheral blood mononuclear cell

PCR	Polymerase chain reaction
PPD	Purified protein derivative
PZA	Pyrazinamide
rBCG	Recombinant bacilli Calmette Guerin
RBC	Red blood cell
RD	Region of difference
RE	Restriction enzyme
RIF	Rifampin
RT	Room temperature
SI	Stimulation index
T_{CM}	T central memory
T_{EM}	T effector memory
Th	T helper
TB	Tuberculosis
TLR	Toll like receptor
TNF	Tumor necrosis factor
TST	Tuberculin skin test
WHO	World Health Organization

LIST OF APPENDICES

CLONING, EXPRESSION AND IMMUNOGENICITY OF RECOMBINANT BACILLE CALMETTE-GUERIN (BCG) CONTAINING T AND B CELL EPITOPES OF *Mycobacterium tuberculosis*

ABSTRACT

Tuberculosis (TB) remains one of the leading causes of morbidity and mortality in humans. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis,* bacille Calmette–Guerin (BCG). However, the efficacy of BCG continues to be debated. The BCG protection against adult pulmonary TB ranged from 0 to 80 % in randomized control trials. In addition, the rising rates of mulit-drug resistant *M. tuberculosis* have worsened the situation. Thus, an improved TB vaccine is urgently needed. Recombinant BCG (rBCG) is one of the most potential approaches in evoking the immune response against TB. In this study, two different types of rBCG were constructed: rBCG expressing T cell epitopes from *M. tuberculosis* Ag85B antigens and Mtb8.4 protein (rBCG018) or a combination of the antigens fused to B cell epitopes from ESAT-6, CFP10 and MTP40 proteins (rBCG032). Polyclonal anti-Mtb8.4 was successfully raised in rabbit and subsequently used for rBCG expression. Immunogenicity study of the vaccine constructs were used for immunization of Balb/c mice. Specific IgG response was obtained against the ESAT-6 and CFP10 in the sera of rBCG032-immunized mice. Splenocytes from these mice showed a high response against the Ag85B antigens and the Mtb8.4 protein, whereas splenocytes from rBCG018-immunized mice elicited a lower response against Ag85B epitopes and a high response against Mtb8.4 protein. Mice immunized with the rBCG strains produced a Th1 pattern of response against the T cell epitopes. Six weeks after the final immunization, the rBCG constructs were recovered from spleen, lung, liver and peritoneal washout. The presences of both constructs in the colonies grown from the organ were detected by PCR. In conclusion, the data obtained from this study demonstrates that T and B epitopes expressed in a single rBCG construct induced

appropriate humoral and cellular immune responses against immunogenic epitopes from *M. tuberculosis*.

PENGKLONAN, EKSPRESI DAN IMUNOGENISITI BACILLE CALMETTE-GUERIN (BCG) REKOMBINAN YANG MENGANDUNGI EPITOP SEL T DAN B *Mycobacterium tuberculosis*

ABSTRAK

Tuberkulosis (TB) merupakan salah satu penyakit yang menyebabkan banyak kematian kepada manusia. Terdapat hanya satu vaksin TB pada masa ini iaitu strain yang telah dilemahkan, *Mycobacterium bovis* bacille Calmette–Guèrin (BCG). Bagaimanapun, keberkesanan BCG masih hebat diperdebatkan. Daripada kajian yang dijalankan secara rawak, keberkesanan BCG terhadap penyakit TB pulmonari dalam kalangan orang dewasa didapati di antara 0 hingga 80 %. Tambahan pula, kadar kerintangan drug TB yang sentiasa meningkat merumitkan lagi masalah yang sedia ada. Oleh itu, vaksin yang lebih berkesan terhadap TB sangat diperlukan. BCG rekombinan (rBCG) merupakan salah satu langkah yang berpotensi untuk meningkatkan sistem keimunan terhadap TB. Di dalam kajian ini, dua jenis rBCG telah dihasilkan: rBCG yang mengkodkan epitop sel T daripada *M. tuberculosis* iaitu antigen Ag85B dan protein Mtb8.4 (rBCG018) atau kombinasi antigen-antigen tersebut dengan epitop sel B iaitu ESAT-6, CFP10 dan protein MTP40 (rBCG032). Anti-Mtb8.4 poliklonal telah berjaya dihasilkan di dalam arnab dan seterusnya digunakan dalam expresi rBCG. Kajian keimunan terhadap konstrak vaksin ini telah dijalankan di dalam mencit Balb/c. Gerakbalas IgG yang spesifik dapat diperhatikan terhadap epitop ESAT-6 dan CFP10 dalam sera mencit yang diimunisasi dengan vaksin rBCG032. Splenosit daripada kumpulan mencit ini juga menunjukkan gerakbalas yang tinggi terhadap epitop Ag85B dan protein Mtb8.4. Splenosit mencit yang diimunisasi dengan vaksin rBCG018 merangsang gerakbalas yang rendah terhadap epitop Ag85B, tetapi menunjukkan tindak balas yang tinggi terhadap protein Mtb8.4. Sel T $CD4^+$ dan $CD8^+$ daripada splenosit mencit yang diimunisasi dengan kedua-dua rBCG menghasilkan gerakbalas Th1 terhadap epitop sel T. Selepas dimunisasi selama 6 minggu, kehadiran

rBCG yang terdapat di dalam limpa, paru-paru, hati dan basuhan peritoneum koloni organ telah dapat dikesan. Sebagai kesimpulan, data yang diperolehi daripada kajian ini menunjukkan bahawa rBCG yang mengkodkan sel T dan B telah berupaya meningkatkan gerakbalas humoral dan sel terhadap epitop-epitop *M. tuberculosis* yang immunogenik.

CHAPTER ONE

INTRODUCTION

1.1 Background

Tuberculosis (TB) remains one of the leading causes of death worldwide (Frieden *et al*., 2003). More than one-third of the world's population have been infected with TB and new infections are occurring at a rate of one per second (WHO, 2006).

1.2 History of TB

TB has been present in humans since ancient times. *Mycobacterium tuberculosis* was first viewed in 1882 when Robert Koch discovered a special staining technique that allowed identifying the organism and announced the discovery of TB bacillus on 24th of March 1882 (Reviewed by Kanai, 1990). The Sanatorium era began after the discovery of the tubercle bacilli by Robert Koch (Reviewed by Bloom and Murray, 1992). In 1982, the first World TB Day was held by World's Health Organization (WHO). Studies have provided evidence for the presence of *M. tuberculosis* complex in ancient skeletal and mummified materials (Crubezy *et al*., 1998; Haas *et al*., 2000; Mays *et al*., 2001; Zink *et al*., 2001). The deoxyribonucleic acid (DNA) analysis of the tissue samples from Egyptian mummies [2125 Before Century (B.C)] has shown that an original *M. tuberculosis* complex was similar to the recent *M. tuberculosis* (Crubezy *et al*., 1998; Zink *et al*., 2003). Brosch *et al.* (2002) reported that *M. africanum*, *M. microti* and *M. bovis* strains diverged from the progenitor of *M. tuberculosis.*

1.3 Global incidence of TB

TB is a major health problem of worldwide concern both in developing as well as in industrialized countries. In 2002, almost 9 million new TB cases occurred (WHO,

2006). One out of 6 adults (15-59 years old) who live in developing countries dies from this disease (Murray *et al*., 1990; Dye *et al*., 1999) and more than 8 million new cases of TB develop every year (Dye *et al*,. 1999). The number of deaths by TB (3 million people) is higher than the number of those caused by heart disease, cancer or any other infectious agents (Murray *et al*., 1992). Moreover, more than 90 % of all TB cases and deaths occur in developing countries (Bloom & Murray, 2003; WHO Fact Sheet, 2003). The incidence of TB has increased significantly in areas with high rates of human immunodeficiency virus (HIV) infection, particularly in sub-Saharan Africa. WHO (WHO Fact Sheet, 2000) reported that the TB incidence rates in this area increased three-fold in the 1990s. It is estimated that most people infected with HIV (90 %) are in developing countries, especially in the 20-35 age group. In some areas, 70 % of new TB patients are HIV co-infected (Elliot *et al*., 1990; De Cock *et al*., 1992). Worldwide, TB is the most common cause of death among acute immunodepression syndrome (AIDS) patients, killing 1 of every 3 of them.

Besides co-infection with HIV, factors that are related to the high TB burden include homelessness and poverty, increasing number of refugees, reduction of governmental support in TB prevention and lack of treatment programs (Brudney & Dobkin, 1991). Another reason for the rising rate of TB cases is due to the high rate of immigration from countries with a high incidence of TB.

TB is the leading infectious cause of death among people in more than 5 years of age in South-East Asia and accounts for approximately 33 % of all the cases of TB in the world. Within South-East Asia, more than 95 % of cases are found in India, Indonesia, Bangladesh, Thailand, and Myanmar (Murray *et al*., 1990; Kochi, 1991; Bloom and Murray, 1992). Frieden *et al*. (2003) reported that 80 % of new TB cases occur in 22 high-burden countries (Figure 1.1).

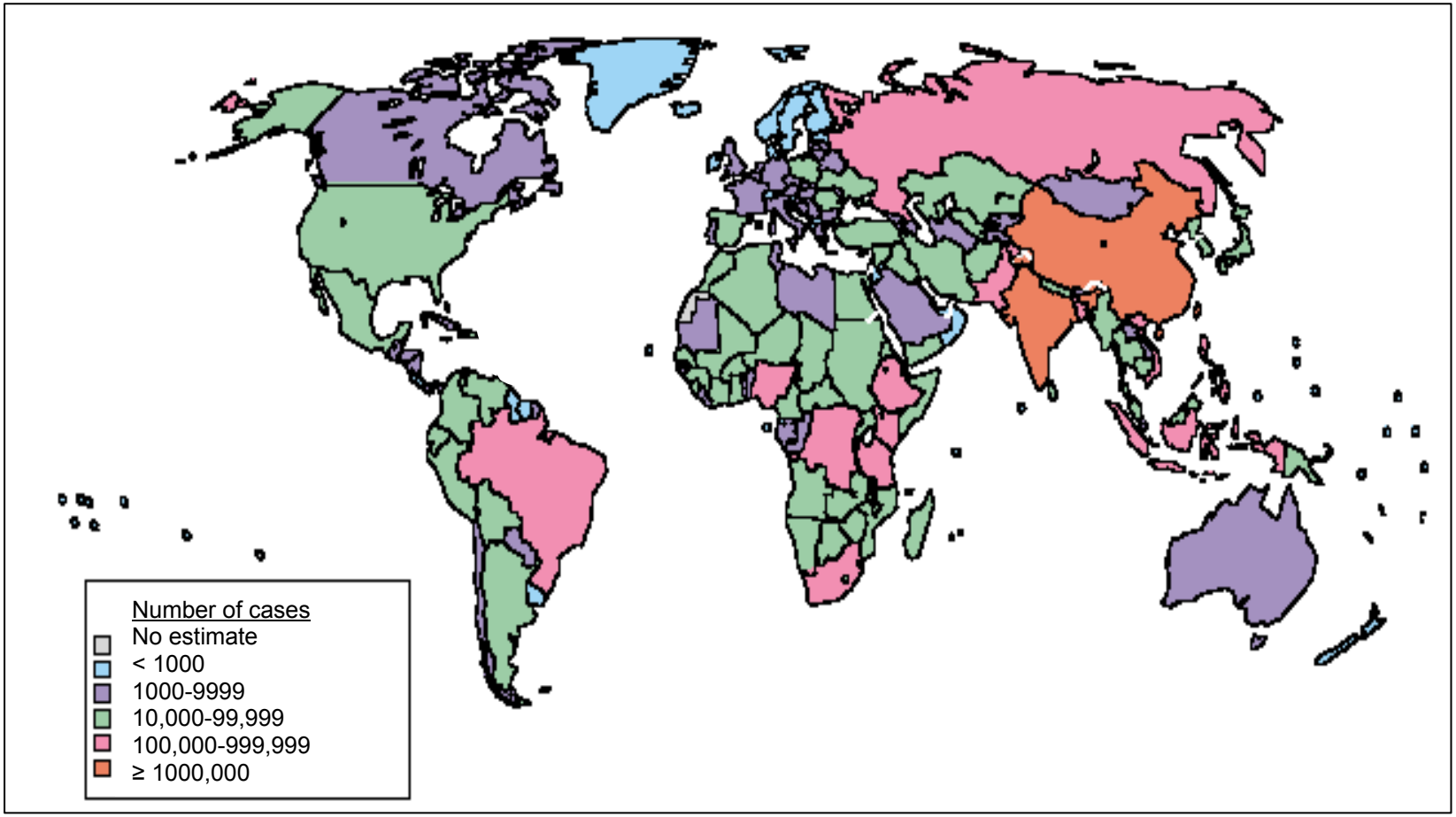

Figure 1.1: TB notification rates in 2001 (Adapted from Frieden *et al*., 2003).

1.4 *M. tuberculosis*

Tubercle bacilli belongs to a distinctive genus of *Mycobacterium*, which include several closely related species of obligate aerobes whose growth is facilitated by carbon deoxide (CO_2). All members of the *Mycobacterium* genus are Gram-positive bacteria. It has a complex cell wall structure that contains unique mycolic acid (Floyd *et al*., 1992). They are non-motile, non-spore forming, straight or slightly curved rod-shaped microbes, 1-4 mm in length and between 0.3-0.6 mm in diameter (Figure 1.2) (Iseman, 2000). The genome of *M. tuberculosis* is 4,411,529 base pairs (bp) long with 3,924 predicted protein-coding sequences.

M. tuberculosis has a high content of guanine (G) and cytosine (C) in its DNA. The high GC content of 65.6 % may be one of the survival strategies employed by the bacteria, since stability of DNA increases directly with number of GC bonds (Cole *et al.*, 1998). *M. tuberculosis* contains granules and vacuoles and grows very slowly doubling its population every 18-24 hours. This characteristic causes delays in diagnosis by culture because the laboratory cultures of clinical materials must be incubated for 2 to 12 weeks to obtain 10^3-10^4 bacteria. Another characteristic that helps *M. tuberculosis* to survive both in the body and in the exterior environment is the resistance to chemicals and drying. They are resistant to drying especially in sputum, where they can remain viable for 6-8 months. They are also resistant to 3 % hydrogen chloride (HCl), 6 % hydrogen sulfate (H_2SO_4) and to 4 % sodium hydroxide (NaOH). The cell wall structure of *M. tuberculosis* is different among prokaryotes and it is the major determinant of virulence. The cell wall contains peptidoglycan and lipid complex (lipids, glycollipids and polysacchatides) (Bloom, 1994). The success of *M. tuberculosis* as a pathogen relies on their ability to survive in the environment and to persist in the host for long periods of time.

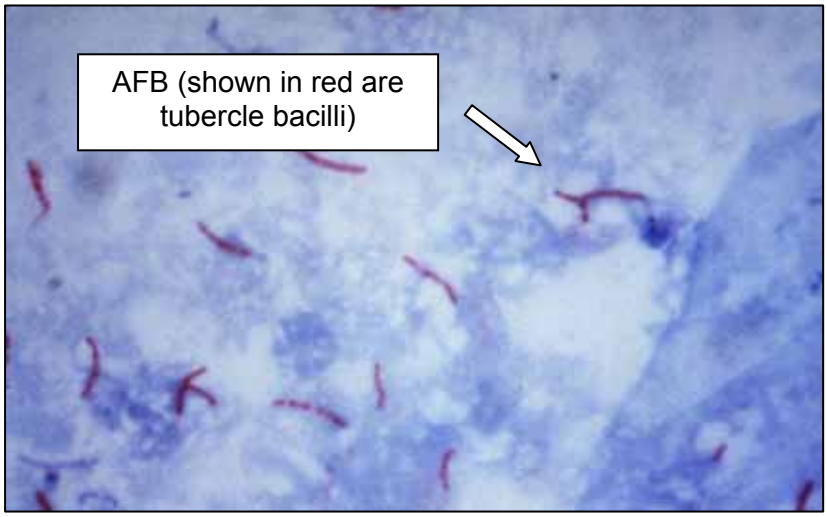

Figure 1.2: AFB smear

1.5 Transmission of *M. tuberculosis*

M. tuberculosis is carried by airborne particles when persons, who have pulmonary TB sneeze, cough, speak or sing. The particles stay for prolonged periods of time in the air. The infectious particles can also be created in other ways either during a procedure to irrigate a TB infected abscess, during laboratory processing of infected tissues or during an autopsy or embalming. Infection occurs when a susceptible person inhales particles containing *M. tuberculosis.* These particles traverse the mouth or nasal passages, upper respiratory tract and bronchi to reach the alveoli of the lungs. Once in the alveoli, the organisms are taken up by alveolar macrophages and spread throughout the body. This will either initiate to active disease or remain dormant and never cause disease (Schluger and Rom, 1998; Frieden *et al*., 2003).

1.6 Pathogenesis of *M. tuberculosis*

Based on the study carried out by van Crevel *et al*. (2002), the chronology of events of TB infection starts with inhalation of *M. tuberculosis* (Figure 1.3). Following inhalation, dendritic cells and macrophages ingest the bacilli and destroy them by phagocytic process (Henderson *et al*., 1997; Thurnher *et al*., 1997).

Alternatively, mycobacteria which escape the initial intracellular destruction will multiply and develop a primary complex consisting of a draining lymph node and this will lead to disruption of the macrophage. The disease may localize (primary TB) and hematogenous dissemination may occur after primary infection. Due to this dissemination, it will cause acute disease (milliary, meningitis TB). At this stage, the blood monocytes, inflammatory cells and dendritic cells are attracted to the lungs. Most often, infection is stabilized at this point and will activated months or years afterwards (post-primary TB or secondary infection). At this stage, the bacilli spread to other parts

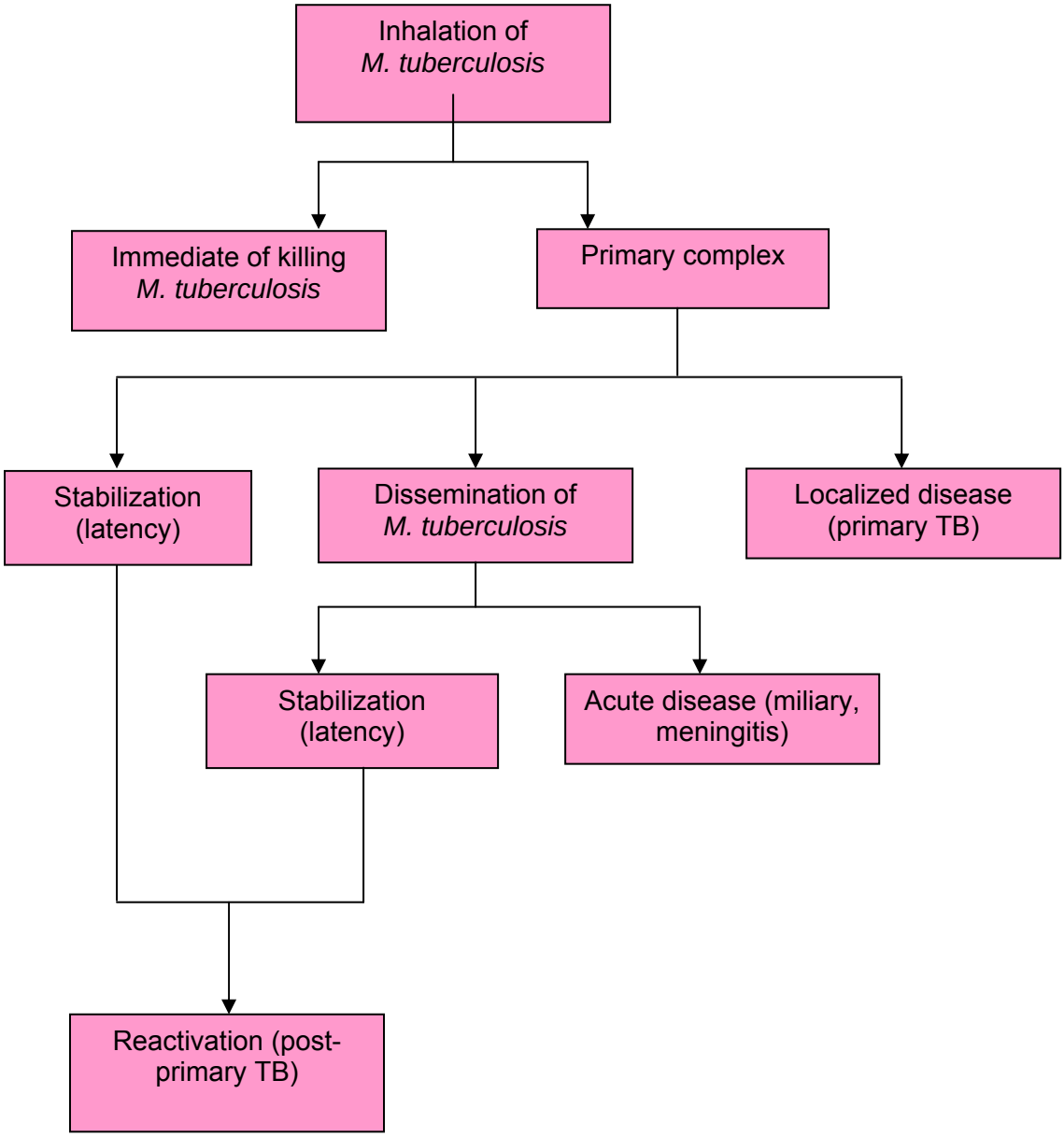

Figure 1.3: The chronology of events of *M. tuberculosis* infection (Modified from van Crevel *et al*., 2002).

of the lungs and organs through the airways which patient will display some of the TB symptoms. In the final stages of TB infection, the organs involved may become damaged and if the patient is not given an appropriate treatment, the re-activation of TB can be fatal.

1.7 Overview of TB disease

TB can infect several organs of the human body such as brain, kidneys, bones and most commonly it affects the lungs which are known as pulmonary TB. It can be divided into two general states: latent TB infection and active TB (pulmonary or extrapulmonary). Extrapulmonary TB contributes for about 20 % of disease in non HIV-infected people but more common in HIV-infected individuals (Shafer & Edlin, 1996). In extrapulmonary TB, the disease may spread to various sites around the body such as the lungs, including the meninges, pericardium, lymph nodes, bones and joints (including spine), genito-urinary tract (including kidneys), intestine, adrenal glands, skin and eyes. Lymph-node TB in the neck is called scrofula and Pott's disease for TB bone (especially the spine). In the spine, the most common location is the thoracic section. Iseman (2000) reported that involvement of the vertebral-body can be followed by disease of an adjacent invertebral disc.

1.8 TB in Malaysia

The state with the highest disease burden is Sabah (143.7 cases/ 100,000 population) 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). In terms of age-distribution of the cases, the majority of cases in Malaysia are in the 15-54 year age group with 67.7 %, 29.5 % for more than 55 years age group and only 2.8 % cases for the childhood TB (Iyawoo, 2004). About 10 % of TB cases in Malaysia are discovered

among the immigrant population. The immigrants normally come from high TB burden neighbouring countries like Indonesia (45.9 %), Philippines (43.5 %), Bangladesh (3.6 %), Myanmar (2.0 %) and Thailand (1.9 %) (Iyawoo, 2004). The increasing number of TB cases in Malaysia is also contributed by the increasing number of HIV infection especially in drug addicts (Mohammad *et al*., 2002). The last factor is due to the lack of knowledge on cleanliness. TB control programme in Malaysia is performed by bacille Calmette-Guerin (BCG) immunization of all newborns and drug treatment of TB patients.

1.9 TB in children

The standard age categories by WHO for TB disease in children are those younger than 15 years old. WHO estimated that 1.3 million new cases of childhood TB occur each year and 450,000 children die due to the disease (WHO, 1989). Dolin *et al*. (1994) reported that there was an estimated 7.5 million total TB cases, of which 650,000 (9 %) occurred in children. Childhood TB appears to be increasing in many parts of the world (Nelson & Wells, 2004). In many industrialized countries, this situation is associated with new immigration patterns (Bibi *et al.*, 1997; Nelson *et al.*, 2004). In many countries, this trend can be associated with many factors including poor economic situation and public health infrastructure, rising rates of HIV infection, difficulty of diagnosing childhood TB and the difficulty of diagnosing HIV-associated pulmonary infections in children. Several studies have reported that poverty, crowding and malnutrition are related with childhood TB (Qazi *et al*., 1998; van Rie *et al*., 1999). Some studies conducted in children have demonstrated that the rates of TB in children infected with HIV ranges from 1 to 19 % in areas with high prevalence of HIV infection (Lucas *et al*., 1996; Drut *et al*., 1997; Ikeogo *et al*., 1997; Chintu *et al*., 2002).

1.10 Symptoms of TB

People with latent TB will have no symptoms because they are in inactive state of disease. However, people with TB disease may have any or all of the following symptoms; cough, feeling tired most of the time, loss of appetite and weight, fever and night sweats. As the illness progresses, people may cough up blood (hemoptysis) and develop severe breathing problems. The symptoms of extrapulmonary 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. In bone disease, the spine is typically involved and it usually causes back pain lasting weeks to months. Sometimes, the disease can progress to involve the spinal cord, causing paralysis. *M. tuberculosis* can also infect the joints, with slowly progressive swelling and pain, usually in a large joint such as the knee.

1.11 Diagnosis of TB

Fast and accurate diagnosis is a very important element of global health measurement to control any diseases. There are some diagnostic techniques that have been developed in order to detect TB.

1.11.1 Chest radiograph

The chest radiograph is an important tool in the diagnosis of pulmonary TB. Pulmonary TB typically demonstrates a number of abnormalities on chest radiographs. Primary pulmonary TB most commonly reveals hilar lymphadenopathy, which may cause right middle lobe compression and atelectasis (American Thoracic Society, 2000a). Pulmonary infiltrates may be seen in primary TB, typically in the middle or lower lobes, but cavitary lesions are uncommon (American Thoracic Society, 2000a). Chest radiograph may be normal in patient with culture proven TB, especially in immunosuppressed patients (Marciniuk *et al*., 1999).

1.11.2 Sputum test

The standard in TB diagnosis is clinical examination, combined with the direct microscopic examination of sputum and culture of bacteria. A patient suggestive of pulmonary TB should have the sputum test on mycobacterial strains by microscopic examination and cultures. Results of *M. tuberculosis* culture can be 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.

1.11.3 Mantoux Tuberculin Skin Test (TST)

The Mantoux TST is the only preferred method of testing patients for latent TB infections. It has been used for the past 50 years to support diagnosis of TB (Huebner *et al*., 1993). An intradermal injection of 0.1 ml purified protein derivative (PPD), which contains 5 tuberculin units, is applied onto the forearm. The interpretation of the test is based on the diameter of induration and not the diameter of erythema. A reaction equal to 5 mm is considered positive in HIV-positive patients, 10 mm is considered positive in people from high-prevalence countries and injection drug abusers. The healthy adults are considered positive if the diameter is equal to or greater than 15 mm (American Thoracic Society, 2000b). However, this test gives poor specificity due to the response produce by BCG vaccination and exposure to non-TB mycobacteria which are similar to the response induced by infection with *M. tuberculosis* (Anderson *et al*., 2000).

1.11.4 Nucleic acid amplification (NAA) test

NAA test is carried out to distinguish between *M. tuberculosis* and *M. avium*. Schluger (2001) recommended the use of nucleic acid amplification test to rapidly diagnose TB patients whose mycobacterial smear is positive.

1.11.5 Interferon-gamma (IFN-γ□) assay

IFN-γ assay has been developed to replace the TST. They operate on a T-cell based approach as TB infection evokes a strong T helper 1 (Th1) type cell-mediated immune response (Munk *et al*., 1997). IFN-□ γ assay is currently not in widespread use since it is very expensive. The assay is based on PPD and region of difference-1 (RD-1) specific antigens are able to discriminate between *M. tuberculosis* and *M. avium* intracellular complex infection (Streeton *et al*., 1998). Moreover, *in-vivo* and *in-vitro* experiments have shown that the IFN-□ γ based on the RD-1 specific antigens have higher sensitivity and specificity than PPD in diagnosis of TB infection (van Pinxteren *et al*., 2000b; Arend *et al*., 2001; Brock *et al*., 2001).

1.12 Treatment of TB

TB can be treated by chemotherapy and this approach has been 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) (reviewed by Friedman, 2000). The modern short course chemotherapy consists of 2 months PZA, 6 months INH and RIF. ETH is added if multi drug resistant-TB (MDR-TB) is suspected (American Thoracic Society, 1993b). 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 (Mitchinson, 2005). RIF is an important agent in the treatment of TB and has bactericidal properties against both intracellular and extracellular *M. tuberculosis* (Friedman, 2000). ETH inhibits the synthesis of arabinogalactan and interrupts the mycobacterial cell wall synthesis (Mikusova *et al*., 1995). In the late 1970s, TB drug development stopped as it was

assumed that the effective drug and the combination with BCG vaccination programmes would lead to the prevention of TB as a public health threat. Unfortunately, more than two-thirds of TB patients do not receive an effective control of TB treatment in certain areas. Nachega & Chaisson (2003) reported that this phenomenon resulted in the MDR-TB defined as to *M tuberculosis* 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.

1.13 Immunity to TB

The human defense system consists of the innate and adaptive immune system. These immune systems synergize to activate and execute an immune response against the infection. The immune response to *M. tuberculosis* is complex and incompletely understood because *M. tuberculosis* is an intracellular pathogenic bacterium which has sophisticated mechanisms to survive inside host mononuclear phagocytic cells (Coler *et al*., 2001).

1.13.1 Innate immunity

Responses independent of lymphocytes have been termed natural immunity, innate immunity or T-independent responses (Bancroft *et al*., 1991; Fearon & Locksley, 1996; Medzhitov & Janeway, 1998). Their responses to pathogens are fast and immediate, which has led to the concept that the innate response is the initial step in the host response to initiate early defense during the primary infection.

The innate response includes the response of various leukocytes, in particular the cells of the mononuclear phagocyte system (the macrophage and dendritic cell), ganulocytes and natural killer (NK) cells. Phagocytic cells play a key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and

expression of co-stimulatory signals and cytokines. When *M. tuberculosis* first enters the alveolar macrophage, phagocytosis of *M. tuberculosis* becomes the first action taken as a response against this infectious agent. The cells involved in the phagocytosis process are dendritic cells, alveolar type II pneumocytes and monocyte-derived macrophage (Bermudez & Goodman, 1996; Herdensen *et al*., 1997; Thurnher *et al*., 1997; van Crevel *et al*., 2002).

Another crucial step in an effective host innate response is the recognition of *M. tuberculosis* or mycobacterial products on macrophage and dendritic cells by toll-like receptor (TLR) (Belvin & Anderson, 1996; Medzhitov *et al*., 1997; Visintin *et al*., 2001). TLR is an important connection between innate and adaptive immune defenses against microbial pathogens. The TLR family comprises of 11 members. The TLR11 has been discovered to be critical in the control of uropathogenic bacteria (Quesniaux *et al*., 2004; Zhang *et al*., 2004). TLR2 is necessary for signaling of the mycobacterial lipoarabinomannan (LAM) (Means *et al*., 1999b; Underhill *et al*., 1999) and a 19 kDa *M. tuberculosis* lipoprotein (Brightbill *et al*., 1999; Noss *et al*., 2001). Its co-receptor, TLR6 is able to control chronic TB infection (Nicolle *et al*., 2004). TLR4 may have a role in controlling TB infection and functions for the fine tuning of inflammation in chronic infections (Fremond *et al*., 2003). After the recognition of *M. tuberculosis* by TLRs, common signaling pathways lead to cell activation and production of cytokines which induces further activation.

Host recognition of *M. tuberculosis* 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 defense against *M. tuberculosis* infection (Belosevic *et al*., 1989; Sher & 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 & 1997) and in IFN-γR knock out (KO) mice (Cooper *et al*., 1993; Flynn *et al*., 1993). In humans, IFN-γ alone is insufficient to enhance the ability of macrophages to control the *M. tuberculosis* infection. Thus, other cytokines are important in working synergistically to decrease the infection caused by *M. tuberculosis.*

Another cytokine is tumor necrosis factor-α (TNF-α) which is produced by the stimulation of T cells, macrophages and dendritic cells (Valone *et al*., 1988; Barnes *et al*., 1993; Henderson *et al*., 1997; Ladel *et al*., 1997; Serbina & Flynn, 1999). TNF-α plays a key role in granuloma formation. In TB patients, TNF-α is detected at the site of disease (Barnes *et al*., 1993; Law *et al*., 1996; Casarini *et al*., 1999). Interleukin (IL) -1β (IL-1 β) is expressed in excess (Schauf *et al*., 1993) and at the site of disease (Law *et al*., 1996; Bergeron *et al*., 1997). Studies in mice suggested that IL-1 β plays an important role in TB disease (Juffermans *et al*., 2000; Yamada *et al*., 2000). IL-12 is also one of the crucial cytokines in controlling *M. tuberculosis* infection and induced following phagocytosis of *M. tuberculosis* by macrophages (Henderson *et al*., 1997; Ladel *et al*., 1997). Lowrie *et al*. (1999) showed that administration of IL-12 DNA can reduce the bacterial numbers in mice with chronic *M. tuberculosis* infection. Besides IL-12, IL-18 and IL-15 are also important in controlling *M. tuberculosis* infection. IL-15 (Berg *et al*., 2002) and IL-18 (Berg *et al*., 2003) have been shown to increase the number of IFN-γ secreted by $CD8^+$ T cells.

1.13.2 Adaptive immunity

1.13.2.1 Humoral immune response

Humoral immune response is mediated by antibodies produced by activated B lymphocytes (plasma cells) and provides the most effective response against extracellular bacteria. These antibodies bind specifically to the antigens thereby eliciting the immune response. The immune system then neutralizes or eliminates them

from the body through ingestion and degradation of the antibody-antigen complex by phagocytes or by cell lysis (Abbas & Lichtman, 2001). During the primary infection of TB, IgM antibody response is activated to nonspecific polysaccharide antigens. The responses develop early but never reach high titer and are insufficient to eliminate the TB infection. Studies regarding humoral response in TB were focused on the development of diagnostic assays (Alifano *et al*., 1997; Laal *et al*., 1997). Demkow *et al*. (2002) developed an IgG assay against the 38 kDa and 16 kDa of *M. tuberculosis* recombinant mycobacterial antigens for the diagnosis of bone and joint TB.

1.13.2.2 Cellular immune response

Cell-mediated immune response is an important response to control *M. tuberculosis* infection. This response provides a major protective immune response to *M. tuberculosis* rather than the humoral responses (Kaufmann, 1995; Feng *et al*., 1999). $CD4^+$ T lymphocytes are central to the human immune response to *M. tuberculosis* infection (Boom, 1996), but supported by other T cell subsets such as $CD8^+$ cytolytic T lymphocytes (CTL) and unconventional T cells ($\gamma\delta$ and $CD1^+$ T cells) (Boom, 1996).

$CD4^+$ T cells recognize antigens in the context of the major histocompatibility complex (MHC) class II molecules, whereas, $CD8^+$ recognizes antigens presented by MHC class I molecules. In general, $CD4^+$ is responsible for directing an immune response by activating effector cells, whereas $CD8^+$ is the killer cells that lyse the infected cells by producing various cytokines such as IFN-γ and TNF-α (Schluger & Rom, 1998). Together, these two types of effector T lymphocytes play a critical role in controlling microbial infections. Several studies reported that $CD4^+$, $CD8^+$ and $\gamma\delta$ T cells are all considered to play a crucial role in controlling TB infection in cattle (Cassidy *et al*., 2001; Walravens *et al*., 2002; Vesosky *et al*., 2004).

Activated $CD4^+$ T cells can differentiate into either Th1 or Th2 cells that secrete specific cytokines. In general, Th1 cells secrete IFN-γ and TNF-α, whereas Th2 cells secrete IL-4, IL-5, IL-10 and IL-13. Th1 response is associated with a strong cell-mediated $CD8^+$ T cell response whereas Th2 response is characterized by a humoral or antibody-mediated immune response (Lucey *et al*., 1996). The activation of Th1 cells by *M. tuberculosis* antigens result in cytokine production, mainly IL-2 and IFN-γ. These cytokines will activate macrophages and other T cells ($CD8^+$ and $\gamma\delta$ T-cell) to provide strong protection against bacterial infection (Grange *et al*., 1995; Feng *et al*., 1999; Flynn & Chan, 2001; Raupach & Kaufmann, 2001).

In general, $CD8^+$ T cell uses two major mechanisms of lysis in order to provide protection against *M. tuberculosis.* $CD8^+$ expresses perforin and granzymes to lyse the infected cells. In humans, $CD8^+$ T cell produces granulysin that is toxic to *M. tuberculosis* and lyse the infected cells (Stenger *et al*., 1997 & 1998). Another mechanism to lyse the target cells is through the Fas/FasL pathway (Clark *et al*., 1995). In addition to killing an infected cell, $CD8^+$ T cells mediate anti-microbial effector functions by secreting anti-microbial and cytokines such as IFN-γ and TNF-α (Herbein, 1997).

$\gamma\delta$ and $CD1^+$ T cells also produce IFN-γ which activate anti-mycobacterial activities in macrophages and effect the mycobacterial growth (Raupach & Kaufmann, 2001). This activation is supported by TNF-α. Tsukaguchi *et al*. (1995) demonstrated that $\gamma\delta$ T cells have the capacity to display cytotoxicity and produce IFN-γ in response to *M. tuberculosis* infected macrophages. A study performed by D'Souza *et al*. (1997) reported that $\gamma\delta$ T cells contribute in the early stages of disease. As the disease become chronic, apoptosis mediated by Fas/FasL clears the majority of *M. tuberculosis* reactive $\gamma\delta$ T cells (Li *et al*., 1998). Raja (2004) suggested that $\gamma\delta$ T cell is important in

patients with latent infection. Studies performed in a primate model demonstrated that the number and activity of γδ T cells increased during the 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 lung. CD1 seems to play a significant role in immune responses to infectious diseases such as TB and some types of uncontrolled cell growth (Brigl *et al*., 2004). In humans, CD1a, b, and c have been associated with effective immune response to leprosy and mycobacterial infections. Brigl *et al*. (2004) also showed that CD1 presentations are being downregulated following exposure to live *M. tuberculosis*.

1.14 Vaccination and memory T cells

TB infection and vaccination usually induce memory T cell. After vaccination, dendritic cells take up the microbial antigens and carry them to adjacent lymph nodes. In the lymph nodes, antigen-presenting dendritic cells that have migrated from the peripheral sites of infection will activate naïve T cells to differentiate into primary effector T cells (Figure 1.4) (Esser *et al*., 2003). During the activation phase, effector T cells can expand to more than 1000-fold within a few days (Goldrath & Bevan, 1999). These memory T cells express effector proteins such as perforin (Opferman *et al*., 1999), granzyme B (Jacob & Baltimore, 1999) and cytokines (Saparov *et al*., 1999). After the activation stage, more than 90 % of the effector T cells die via apoptosis (Lin *et al*., 2000). This phenomenon is important in order to maintain the homeostasis and prevent autoimmunity (Lenardo *et al*., 1999). A few effector T cells become long-lasting 'memory' T cells which retained their antigen specificity and can persist for a lifetime in the absence of antigen and even MHC molecules (Zinkernagel, 2000; Sprent & Surh, 2001).

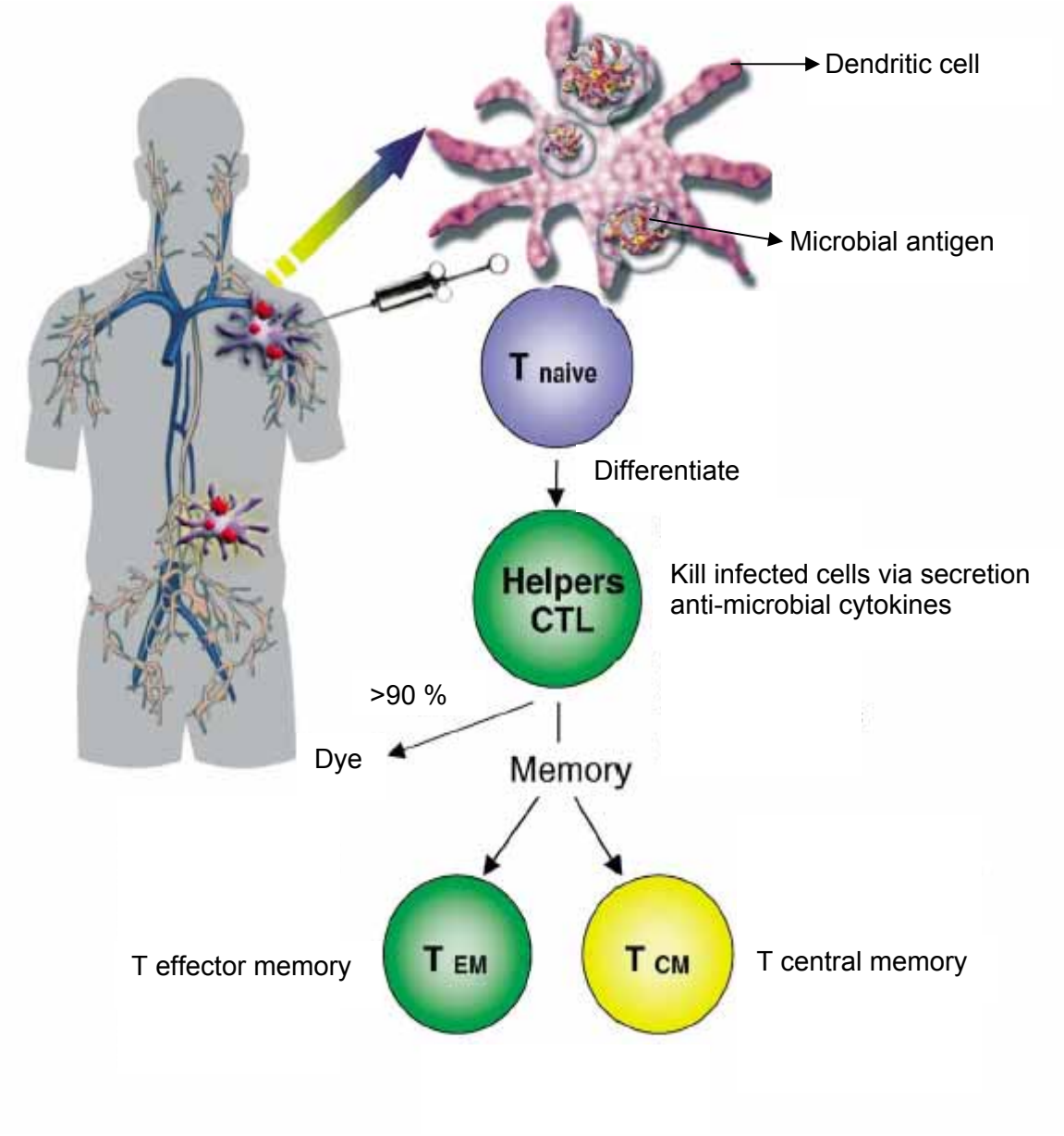

Figure 1.4: Vaccine induction of memory T cells in humans. (Modified from Esser *et al*., 2003).

'Memory' in the T-cell compartment results from the presence of memory T cells, which are found at high frequencies and can respond faster and more efficiently than naïve T cells (Kedl & Mescher, 1998; Bachmann *et al*., 1999; Cho *et al*., 1999; Zimmermann *et al*., 1999). Memory T cells can be divided into the central memory (T_{CM} – derived from lymph node) and effector memory (T_{EM} – derived from peripheral tissues) subsets which can proliferate in response to an antigen or cytokines and perform effector functions (Sallusto *et al*., 2004). T_{CM} are involved in secondary responses and long term protection whereas T_{EM} are involved in immediate protection.

1.15 BCG

BCG was developed by Albert Calmette and Camille Guerin at the Institute Pasteur in Lille, France between 1908 and 1920 from a virulent *M. bovis* strain via 231 serial passages. Studies have shown that during *in vitro* passage, *M. bovis* became attenuated due to loss or rearrangement of several gene complexes (Mahairas *et al*., 1996; Behr et al., 1999; Gordon *et al*., 1999; Brosch *et al*., 2001). BCG is the oldest vaccine currently used throughout the world over the century (Starke, 1991; Huebner, 1996). It is believed that more than 3 billion doses of the vaccine have been administered worldwide (Fine, 1989). Vaccination with BCG still remains the standard for TB prevention in most countries because of its efficacy in preventing TB meningitis.

1.15.1 Efficacy and effectiveness of BCG

BCG is inexpensive, requires only one encounter with the baby and has minor side effects. Besides its wide usage as a vaccine against TB, BCG is also used as immunotherapy against intravesicular bladder cancer (Alexandroff *et al*., 1999). BCG is also a promising vehicle for delivery of heterologous antigens (Kaufmann & Andersen, 1998). Such BCG-based carrier strains have been evaluated for the immunization against viral (Leung *et al*., 2000), bacterial (Abomoelak *et al*., 1999; Hayward *et al*.,

1999) and parasitic pathogens (Matsumoto *et al*., 1998; Streit *et al*., 2000) as well as in anti-tumor vaccination models (Duda *et al*., 1995).

However, its efficacy varies widely from 0 to 80 % in different populations. The low efficacy is consistent in many tropical regions of the world where the vaccine is most needed (Figure 1.5) (Fine, 1989; 1995). Lagranderie *et al*. (1996) reported that the differences of BCG strains, incorrect preparation of the freeze-dried vaccine, the age at vaccination and the methodological differences are important factors that contribute to the variable efficacy of the vaccine. However, the main reason for the failure of BCG is due to the exposure to non-TB mycobacteria which can block the multiplication of BCG. Brandt *et al*. (2002) reported that this exposure can prevent the induction of an efficient BCG-mediated immune response and give less protection against TB. The study also concluded that BCG is sensitive to the influence of preexisting immune responses to antigens shared with certain environmental strains. The efficacy of BCG vaccination also appears to vary with geographical latitude. The farther from the equator; the efficacy of the vaccine is higher (Brandt *et al*., 2002). Norazmi *et al*. (2005) reported that the failure of BCG may be due to the absence of important T cell antigens in BCG which are important in evoking the immune system against *M. tuberculosis.*

1.16 Development of effective vaccines against TB

The development of more effective TB vaccines include pre-infection vaccines to prevent primary infection, post-infection vaccines that inhibit TB progression in recently exposed individuals and therapeutic vaccines for MDR-TB patients (Ginsberg, 2002). Moreover, a vaccine against latent TB infection is urgently needed to cure the 2 billion individuals who are already infected (Dye, 2004). In order to generate an effective vaccine, some approaches have been developed including living and non-living putative TB vaccines that are better than the parent vaccine (Orme, 1999; McMurray, 2000; Andersen *et al*., 2001).

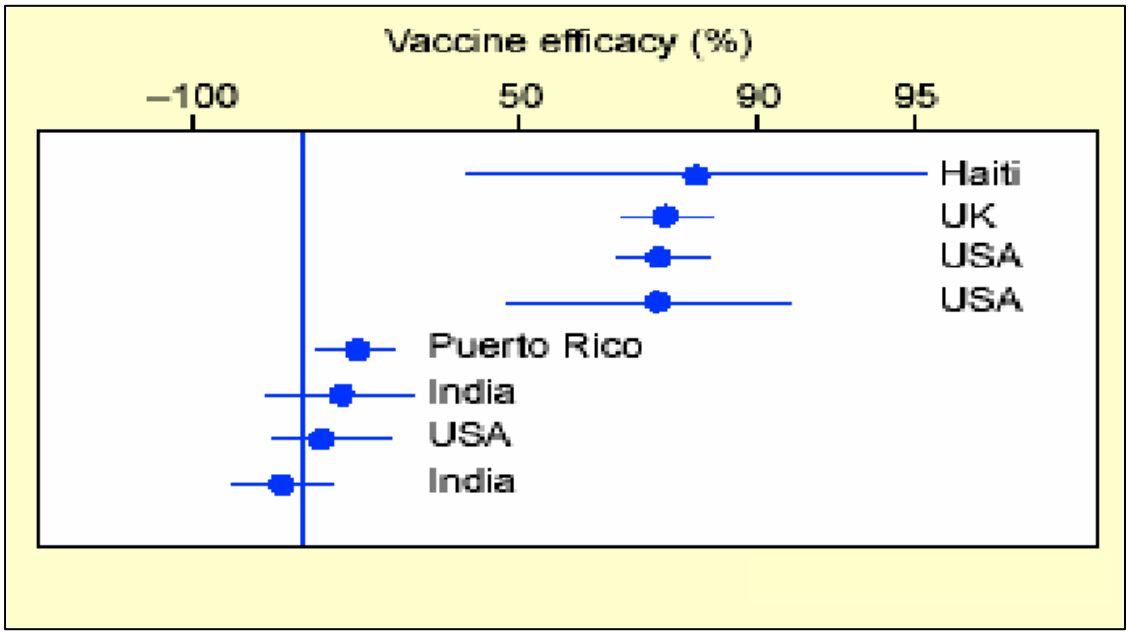

Figure 1.5: Results of various controlled clinical trials of BCG vaccine (Adapted from Orme, 1999).

1.16.1 Live vaccines

1.16.1.1 Attenuated mycobacteria

Attenuation aims to minimize the virulence of a pathogen. The attenuated mycobacteria can elicit protective immune responses, but does not cause any clinical disease. A number of *M. tuberculosis*, *M. bovis* and *M. bovis* BCG auxotrophs have been constructed with mutation in certain metabolic pathways (Guilera *et al*., 1996; Berthet *et al*., 1998a; Jackson *et al*., 1999). Mutant strains of *M. tuberculosis* have been constructed with mutations in antigen 85A (Ag85A) and Ag85B. These mutants showed slower growth rate within human and murine monocyte cell lines (Armitige *et al*., 2000). These efforts may lead to the generation of a more effective vaccine and to a better understanding of the biology of mycobacterial infection.

1.16.1.2 Recombinant BCG (rBCG)

rBCG techniques may be useful for the development of a more effective TB vaccine than the parental BCG strain (Ohara *et al*., 2001). The rBCG has been suggested to protect against TB meningitis (Colditz *et al*., 1994) and also able to minimize the dissemination of the bacteria from the primary lesion to other parts of the lungs (Orme, 1999). Recent studies carried out by Kawahara in 2006 indicated that their constructed rBCG-SIVGag has the ability to effectively induce long-lasting, cell-mediated and humoral immunity against both viral and bacterial antigens in guinea pigs. These results suggest that rBCG-Gag has the potential to elicit specific immunity not only for TB but also for HIV.

1.16.2 Non-live vaccines

1.16. 2.1 Subunit vaccine

A number of subunit vaccines which are based on various secreted and non-secreted protein (lipid or carbohydrate) components of *M. tuberculosis* have been studied (Andersen, 1994; Horwitz *et al*., 1995; Roberts *et al*., 1995). Brandt and

colleagues (2002) suggested that the subunit vaccine may be much less influenced by prior contact with environmental mycobacteria and therefore more protective in preventing the disease. However, these non-living subunit vaccines still failed to provide better protection than BCG. Furthermore, the main disadvantage of subunit vaccines is that they are expensive to produce and require multiple boosters.

1.16.2.2 DNA vaccine

Another approach is the use of DNA vaccine encoding mycobacteria proteins incorporated into the plasmid. The plasmid contains a mammalian promoter that is injected into the muscle. Many researches have focused on the secreted antigens and heat-shock proteins (hsp) encoded in the DNA (Huygen *et al*., 1996; Tascon *et al*., 1996; Baldwin *et al*., 1998; Lowrie *et al*., 1999). Vaccination with DNA encoding Ag85A (Huygen *et al*., 1996; Baldwin *et al*., 1998) and the 6-kDa early secretory antigenic target (ESAT-6) (Brandt *et al*., 1996; Kamath *et al*., 1999; Brandt *et al*., 2000) have been shown to induce significant protection against TB.

1.16.3 Prime boost vaccination

The prime-boost approach is based on the concept of administration of the same mycobacterial antigen in order to gain a higher level of immune response. This approach involves two approaches; homologous and heterologous boosting. Homologous boosting refers to the antigens being delivered by the same delivery system with each subsequent dose (McShane *et al*., 2001; 2004; McShane & Hill, 2005). Heterologous boosting refers to the administration of the same mycobacteria antigens delivered by different vectors (McShane & Hill, 2005). It has been suggested that a combination of rBCG and DNA vaccines expressing various epitopes of *M. tuberculosis* administered in a prime-boost manner could be one of the best approaches to control TB infection (Norazmi & Musa, 2004; McShane & Hill, 2005)