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

by

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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...

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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.

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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
γδ	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

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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).

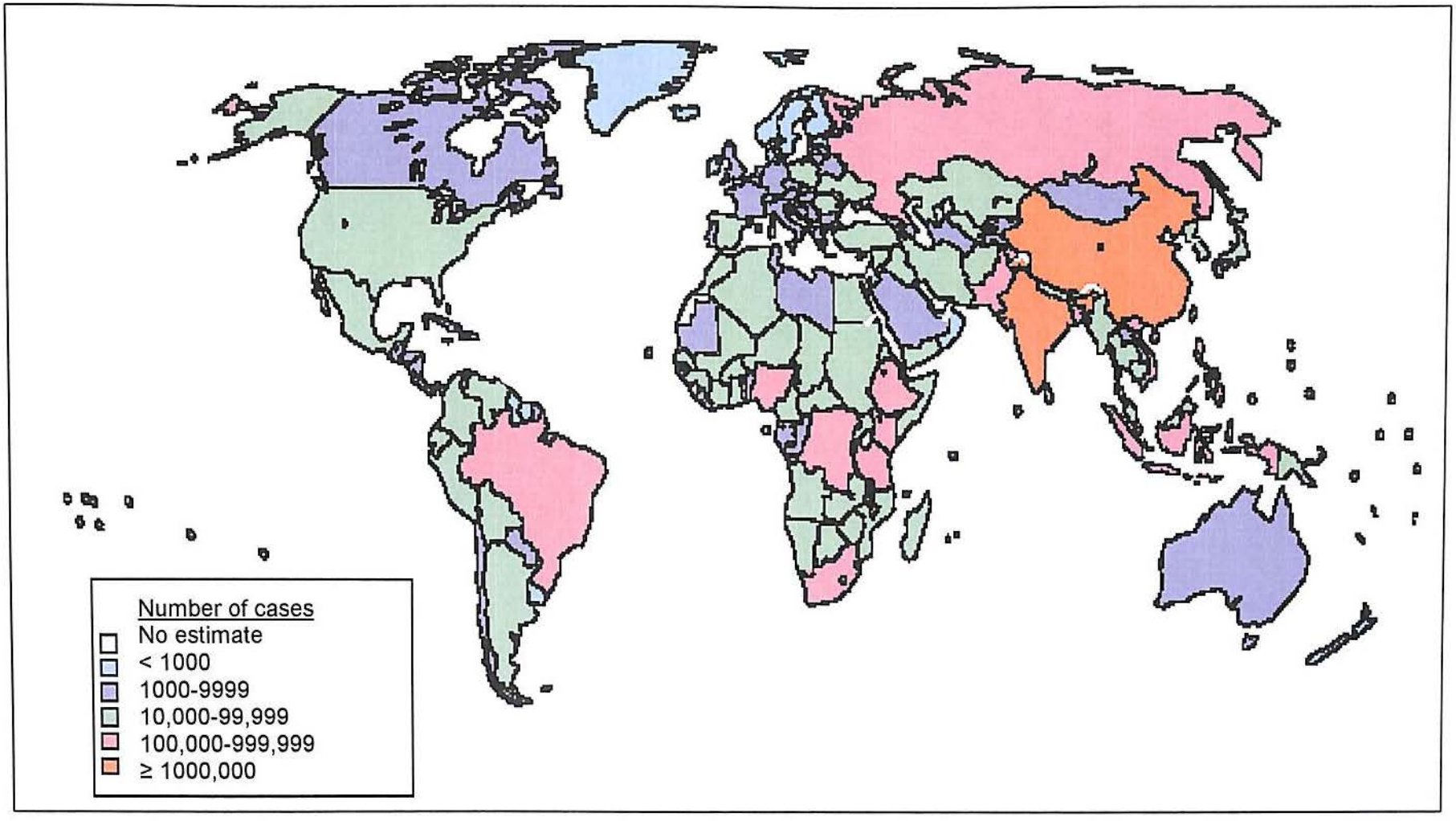

Figure 1.1: TB notification rates in 2001 (Adapted from Frieden *et al.*, 2003).