DETERMINATION OF HUMORAL IMMUNE RESPONSE IN MICE IMMUNISED WITH MILK EXPRESSING THREE-TB EPITOPES

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by

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DECLARATION

I hereby declare this dissertation is the result of my own investigations, except otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use this dissertation for teaching, research, and promotional purposes.

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(PATRICIA JANE A/P WILFRED MARTIN)

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µL	Microliter
mL	Milliliter
µg/mL	Microgram per milliliter
2EHRZ	2 months of Ethambutol, Isoniazid, Rifampicin, and Pyrazinamide
4HR	4 months of Isoniazid and Rifampicin
Acr	Alpha crystallin protein
AFB	Acid-fast bacilli
Ag85B	Antigen 85B protein
ART	Antiretroviral therapy
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
CPA	Cross-Priming Amplification
DC	Dendritic cell
DM	Diabetes Mellitus
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DOT	Directly Observed Treatment
DST	Drug Susceptibility Testing

ELISA	Enzyme Linked Immunosorbent Assay
EMB	Ethambutol
EPI	Expanded Programme of Immunization
EPTB	Extrapulmonary Tuberculosis
F	F-value
Fn	Fibronectin
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
HspX	Heat shock protein
ID	Intradermal
IGRA	Interferon Gamma Release Assay
INH	Isoniazid
LAMP	Loop-mediated isothermal amplification
LiPA	Line probe assays
LTBI	Latent tuberculosis infection
MALT	Mucosa-associated lymphoid tissue
MGIT	Mycobacteria Growth Indicator Tube
MODS	Microscopic Observation of Drug Susceptibility
MOH	Ministry of Health Malaysia
Mtb	*Mycobacterium tuberculosis*
NTM	Nontuberculous mycobacteria
OD	Optical density
p	p-value

PBS	Phosphate Buffered Saline
PPD	Purified Protein Derivative
PTB	Pulmonary Tuberculosis
PZA	Pyrazinamide
QFT-GIT	QuantiFERON-TB Gold In-Tube Test
RIF	Rifampin
RpfE	Resuscitation Promoting Factor E
sIgA	Secretory Immunoglobulin A
TB	Tuberculosis
TMB	3,3',5,5'- tetramethylbenzidine
TNF	Tumor Necrosis Factor
TST	Tuberculin skin test
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant

Penilaian Gerak Balas Imun Humoral dalam Mencit yang Diimunisasi dengan Susu yang Mengekspres Tiga Epitop TB

ABSTRAK

Tuberkulosis (TB) kekal sebagai salah satu cabaran kesihatan global yang utama, meskipun vaksin Bacillus Calmette-Guérin (BCG) telah digunakan secara meluas. Namun, cabaran vaksin BCG dalam merangsang imuniti mukosa yang kuat, khususnya dalam kalangan orang dewasa, menekankan keperluan mendesak untuk alternatif yang lebih berkesan. Kajian ini meneroka potensi vaksin oral inovatif berasaskan susu yang mengandungi tiga epitope antigen TB. Kajian ini meneliti gerak balas imun humoral yang dihasilkan oleh mencit Balb/c yang diimunisasi dengan susu yang mengandungi tiga epitop antigen TB iaitu Ag85B, Acr, dan RpfE yang diekspreskan dalam susu kambing menggunakan konstruksi fusi IgA sekretori untuk menghasilkan sebuah susu yang mengandungi multi-epitope TB:IgA bagi memperkuatkan imuniti mukosa. Mencit telah diimunisasi dengan lima kumpulan rawatan iaitu Susu Harian (MD), Susu Biasa (NM), BCG sahaja (BCG-O), BCG + Susu Harian (BCG-MD), dan BCG + Susu Biasa (BCG-NM) untuk menilai gerak balas imun terhadap tiga epitop tersebut. Selepas dua minggu imunisasi, sampel serum, air liur, dan cecair BAL dikumpulkan untuk dianalisis. Plat ELISA dilapisi dengan antigen yang tersebut untuk menentukan tahap IgA dan IgG spesifik antigen. Bacaan ketumpatan optik (OD) digunakan untuk menilai gerak balas imun, dan analisis menggunakan statistik dijalankan untuk menentukan perbezaan yang signifikan antara kumpulan rawatan. Kajian ini mendapati bahawa calon vaksin berasaskan susu mampu merangsang gerak balas imun IgG dan IgA spesifik antigen dengan sangat baik, menonjolkan potensinya untuk memberikan

perlindungan imun yang lebih berkesan. Hasil kajian ini menunjukkan bahawa vaksin tersebut berpotensi besar untuk menangani cabaran utama dalam pencegahan TB, khususnya dengan menyasarkan permukaan mukosa yang merupakan tapak utama jangkitan *Mycobacterium tuberculosis*. Kajian ini menonjolkan prospek yang menjanjikan bagi vaksin mukosa oral sebagai strategi pelengkap atau alternatif kepada vaksinasi BCG secara intradermal. Pendekatan ini diyakini dapat memperkukuhkan perlindungan dan kawalan terhadap TB secara lebih berkesan. Namun demikian, penyelidikan lanjutan dan ujian klinikal yang menyeluruh amat diperlukan untuk mengesahkan keberkesanan serta mengoptimumkan pendekatan inovatif ini bagi kegunaan masa hadapan.

Determination of Humoral Immune Response in Mice Immunised with Milk Expressing Three-TB Epitopes

ABSTRACT

Tuberculosis (TB) continues to pose significant global health challenges despite the widespread use of the Bacillus Calmette-Guérin (BCG) vaccine. However, its limitations in inducing robust mucosal immunity, especially in adults, necessitate alternative approaches. This study explores the potential of a novel oral vaccine utilizing milk containing multi-epitope TB antigens. This study investigates the humoral immune response elicited in Balb/c mice immunized with milk containing multi-epitope tuberculosis antigens. These antigens which are Ag85B, Acr, and RpfE were expressed in goat milk with a secretory IgA fusion construct designed to produce milk containing multi-epitope TB:IgA to enhance mucosal immunity. The mices were immunized with five treatment groups which are Milk Daily (MD), Normal Milk (NM), BCG only (BCG-O), BCG + Milk Daily (BCG-MD), and BCG + Normal Milk (BCG-NM) to assess immune responses against three *Mycobacterium tuberculosis* epitopes. Two weeks post-immunization, serum, saliva, and BAL fluid samples were collected for analysis. ELISA plates were coated with the respective antigens to measure antigen-specific IgA and IgG levels. Optical density (OD) readings were used to quantify immune responses, and statistical analysis was conducted to determine significant differences between treatment groups. The immunized mice groups, including BCG and milk combinations, demonstrated varying levels of systemic IgG and mucosal IgA antibodies in serum, saliva, and bronchoalveolar lavage samples. Among the treatment groups, the milk-based vaccine candidate elicited robust antigen-specific IgG and IgA responses, indicating

its potential for providing targeted immunity. These findings indicate the vaccine's potential to address key challenges of TB prevention, particularly in targeting mucosal surfaces which is the primary site of *Mycobacterium tuberculosis* infection. This study highlights the promise of oral mucosal vaccines as a complementary or alternative strategy to intradermal BCG vaccination, aiming to enhance protection and control TB more effectively. Future research and clinical trials are needed to validate these findings and further optimize this innovative vaccine approach.

CHAPTER ONE: INTRODUCTION

1.1 Research Background

Tuberculosis is an infectious bacterial infection caused by the bacteria, *Mycobacterium tuberculosis* (*Mtb*), that primarily affects the lungs of the infected person and may also affects other parts of the body such as kidneys, brain or the spine. Globally, TB ranks as the second foremost infectious cause of mortality subsequent to COVID-19, surpassing the death toll attributed to HIV (Human Immunodeficiency Virus) and AIDS. Tuberculosis is endemic in every nation and affects individuals of all age demographics. It is noteworthy that TB is both preventable and curable. It is capable of spreading the disease via airborne particles when the infected person coughs, sneezes and spits (World Health Organization, 2023). Despite being a preventable and curable disease, TB is said to be the leading infectious cause of death worldwide. Unfortunately, Malaysia has experienced a marked increase in cases. In 2023, reported TB cases rose to 26,781, compared to 25,391 in 2022 and 21,727 in 2021. Globally, 10 million people contract TB annually (World Health Organization, 2019).

There are two types of TB infections which are active TB and latent TB infections (LTBI). Active tuberculosis disease occurs when the immune system is unable to effectively manage the infection. Pathogens may disseminate throughout the pulmonary system or other regions of the body. The onset of active TB disease may transpire immediately following the initial infection; however, it more commonly manifests after a duration of months or even years following a latent TB infection. The manifestations of active TB disease within the pulmonary system typically commence gradually and may exacerbate over the course of several weeks. Meanwhile, it is common for the primary infection to be evoked by a phase

known as latent tuberculosis infection. Immune system cells construct a barrier encircling pulmonary tissue that harbors tuberculosis pathogens. These pathogens are rendered harmless as long as the immune system maintains their containment. Nevertheless, the pathogens persist in a dormant state. There are no clinical manifestations present during the latent tuberculosis infection phase.

The BCG vaccine is currently the only approved vaccine available for human use against tuberculosis. While its effectiveness remains a topic of debate, the BCG vaccine provides protection for newborns and young children against widespread forms of TB. However, it does not offer effective protection for adults against active TB infections (Monteiro-Maia & De Pinho, 2014). The BCG vaccine is created from a weakened strain of *Mycobacterium bovis*, a bacterium closely related to *M. tuberculosis*, the causative agent of tuberculosis. Developed over 13 years from 1908 to 1921 by French bacteriologists Albert Calmette and Camille Guérin, the vaccine was named Bacillus Calmette-Guérin (BCG). It is primarily administered shortly after birth to infants who are at high risk of tuberculosis. The BCG vaccine elicits an immune response that provides partial protection to infants and young children against severe forms of tuberculosis (World Health Organization, n.d.). In Malaysia, the BCG introduced its National BCG Vaccination Program in 1961, the routine vaccination is provided to the newborns at birth on their upper left arm through intradermal injection (Pantai Hospital, n.d.). The intradermal (ID) administration is the primary method for BCG vaccination globally; however, this approach is unlikely to generate optimal mucosal immunity in the lungs but it produces stronger systemic immune responses.

Mucosal immunity is an important aspect in combating the TB. Oral BCG vaccination generates stronger and more consistent mucosal secretory IgA responses compared to ID

BCG vaccination. For a predominantly pulmonary disease such as TB, mucosal vaccination is an appealing strategy, as it stimulates both localized and systemic immunity. As a pathogen transmitted via mucosal surfaces, *Mtb* infects humans and animals primarily through the respiratory tract through mucosal tissues. Despite serving as a physical barrier against pathogen invasion, the respiratory mucosa primarily functions as the body's first line of defense through the mucosal barrier. Additionally, it acts as an inductive site for mucosal immune responses, which are part of the third line of defense and provide specific immunity against pathogens. Consequently, there is an emphasis on developing new vaccines and innovative delivery methods that are needed so that it directly stimulates respiratory mucosal immunity to achieve improved protection against *Mtb* infection.

1.2 Problem Statement

Despite ongoing efforts, tuberculosis continues to persist, with many cases remaining unresolved globally. According to the World Health Organization (WHO), latent tuberculosis infection is a state of persistent immune response to stimulation by *Mycobacterium tuberculosis* antigens in the absence of clinical signs and symptoms suggestive of TB disease. In recent years, the prevalence of LTBI was estimated to be approximately one-quarter of the world's population. Reactivation takes place when subclinical latent infection progresses into active TB, in other words LTBI represents an important source of re-infection and/or emergence point for new cases with active TB (Kiazyk & Ball, 2017). Therefore, this becomes a reason where the effectiveness of the available vaccine to be limited as it cannot treat the patients with latent TB infection and eventually reduces the overall impact in diminishing the spread of TB especially in regions where TB is highly prevalent. In addition, since *Mycobacterium tuberculosis* (*Mtb*) enters the host via infectious aerosols, the mucosal surfaces are often the initial point of contact with the pathogen. Mucosal immunity can then activate a specific protective immune response within the mucosa-associated lymphoid tissue (MALT), which plays a crucial role in preventing *Mtb* infection. In this case, the currently available TB vaccine, BCG vaccine is capable of generating systemic immunity but not able to produce mucosal immunity in the infected patients. The administration of the BCG vaccine via needle injections presents certain risks, such as the possibility of infections at the injection site and other adverse effects (Alfawaz et al., 2015). While the procedure is typically safe when performed with appropriate medical precautions, there remains a risk of localized bacterial infection if aseptic techniques are not adhered to. Moreover, individuals with

compromised immune systems, such as those with HIV, may face more serious complications, including the potential onset of TB disease.

1.3 Rationale of Study

The vaccine candidate was developed during a previous postgraduate research project in collaboration with the University of Concepcion, Chile. The method for producing the TB vaccine candidate in this study involves developing a multi-epitope gene construct that targets specific TB antigens (Ag85B, Acr/HspX, RpfE). These genes are combined with secretory IgA (sIgA) to strengthen mucosal immunity. This construct is incorporated into adeno-associated viral vectors, which are then transduced into the mammary glands of pregnant goats. Consequently, the goats produce the chimeric TB-sIgA protein in their milk. The milk is purified and administered orally to mice to assess the humoral immune response by measuring antibody levels.

The BCG vaccine, the only licensed and currently available vaccine for tuberculosis, is among the most widely administered vaccines due to its affordability and safety. It is effective in preventing severe disseminated forms of the disease in children and newborns. However, it does not provide effective protection against adult pulmonary tuberculosis (Stylianou et al., 2019). Additionally, the BCG vaccine is contraindicated for infants infected with HIV. Despite the relative effectiveness of BCG in infants, a significant unresolved question is why the vaccine does not prevent pulmonary tuberculosis in adolescents. It is suggested that the immune memory may decline during adolescence, which is a critical period for TB infection and its progression to become an active Tb case. One potential explanation is that immunological memory is established by BCG at a young age whether in neonates or infants when the immune system is still not fully developed. However, many

other factors may also play a role in diminishing BCG efficacy and increasing susceptibility to tuberculosis in young adults (Monteiro-Maia & De Pinho, 2014).

Given that *M. tuberculosis* primarily infects the lungs, aligning the vaccination route with the infection route may enhance the effectiveness of TB vaccines. The oral route could serve as a more efficient booster following an initial respiratory or systemic vaccination. This is because using a mucosal vaccine, it may able to eradicate the infection way more rapidly as it induces mucosal immunity (Stylianou et al., 2019). Recently, mucosal immunization has gained attention in tuberculosis vaccine research due to its ability to provide mucosal protection against all forms of TB infections. It is essential for the route of vaccine administration to correspond with the route of infection, as mucosal vaccines present a more effective strategy for combating tuberculosis infections (Li et al., 2012).

1.4 Objectives

1.4.1 General Objective

I. To determine the humoral immune response of the Balb/c mice immunized with the milk containing multi – epitope TB:IgA.

1.4.2 Specific Objectives

I. To determine the IgG levels in Balb/c mice that is immunized with the milk containing multi – epitope TB:IgA.

II. To determine the IgA levels in the saliva of Balb/c mice immunized with the milk containing multi – epitope TB:IgA.

III. To determine the IgA levels in bronchoalveolar lavage (BAL) sample in Balb/c mice immunized with milk containing multi – epitope TB:IgA.

1.5 Hypothesis

1.5.1 Null Hypothesis (H0)

There is no increase in the humoral immunity in Balb/c mice immunized with milk containing multi – epitope TB:IgA.

1.5.2 Alternative Hypothesis (HA)

There is an increase in the humoral immunity in Balb/c mice immunized with milk containing multi – epitope TB:IgA.

CHAPTER TWO: LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis is a multisystem disease with diverse presentations and manifestations, ranking as the second leading cause of death from infectious diseases worldwide, following COVID-19. The World Health Organization (WHO) estimates that 2 billion people carry latent TB, and in 2021, TB resulted in 1.6 million deaths globally, including 187,000 deaths among individuals with HIV. The disease seems to be prevalent in various regions globally, which is followed by a rising incidence of drug-resistant TB worldwide. Moreover, a coinfection with HIV has significantly contributed to the emergence and spread of drug resistance (Sahra, n.d.). The disease is caused by the bacteria, *Mycobacterium tuberculosis*, for which humans serve as the primary reservoir. Similar diseases may occasionally be caused by closely related mycobacteria, including *M. bovis*, *M. africanum*, and *M. microti*. Collectively, these bacteria, along with *M. tuberculosis* and several other less common mycobacteria, are known as the *Mycobacterium tuberculosis* complex (Nardell, 2022).

Mycobacteria responsible for diseases that may be mistaken for tuberculosis are commonly referred to as nontuberculous mycobacteria (NTM). These organisms can also interfere with laboratory tests designed for tuberculosis diagnosis, leading to potential confusion in test results. NTM cause diseases that might mimic TB but are not spread through human contact. A well-known type of NTM is the *Mycobacterium avium* complex. Conversely, TB, which is highly contagious, spreads through the air when an infected person expels respiratory droplets that contain the bacteria. These particles, which measure approximately 1 to 5 microns in diameter which are less than 1/5000 of an inch that can remain airborne for several

hours, depending on environmental conditions. These airborne particles can remain in the environment for hours, posing a risk of transmission when inhaled by others, which accounts for causing the active TB in humans. The other type of TB infection which is the latent TB infection occurs when tubercle bacilli are present in the body but are kept under control by the immune system. The immune system contains the bacilli by producing specific immune cells that encircle the bacilli, forming a shell that serves as a barrier to prevent their spread. Individuals with latent tuberculosis infection, who do not have active TB disease, are not contagious which means that they are asymptomatic and cannot transmit the infection to others. The majority of these individuals also display normal chest X-ray results (Centers for Disease Control and Prevention, 2019).

2.2 Prevalence of Tuberculosis

2.2.1 Global Prevalence of Tuberculosis

Tuberculosis primarily impacts adults in their most productive years, though people of all ages are at risk. More than 80% of TB cases and deaths occur in low- and middle-income countries. TB is widespread across the globe. Based on Figure 2.1, in the year 2022, the highest number of new cases were reported in the WHO South-East Asia Region (46%), followed by the African Region (23%) and the Western Pacific Region (18%). Around 87% of new cases were concentrated in 30 high-burden countries, with more than two-thirds of the global total found in Bangladesh, China, the Democratic Republic of the Congo, India, Indonesia, Nigeria, Pakistan, and the Philippines. In 2022, it was estimated that 10.6 million individuals globally developed TB, comprising 5.8 million men, 3.5 million women, and 1.3 million children. TB affects all countries and age groups. It is both curable and preventable.

Those with weakened immune systems, such as individuals with HIV, malnutrition, diabetes, or those who use tobacco, are at higher risk of developing TB. In 2022, 2.2 million new TB cases were linked to undernutrition, 0.89 million to HIV, 0.73 million to alcohol use disorders, 0.70 million to smoking, and 0.37 million to diabetes (World Health Organization, 2023). In 2023, the WHO convened a guideline development group to evaluate the use of targeted next-generation sequencing for detecting drug-resistant TB directly from sputum samples. A rapid communication was subsequently issued to emphasize the key findings of this assessment. This category of tests represents a significant advancement toward comprehensive drug susceptibility testing (DST). Significant progress in reducing the global tuberculosis burden was made until 2019, but the COVID-19 pandemic caused a major setback, leading to reduced access to TB care in 2020 and only partial recovery by 2021. This resulted in increased TB cases and deaths for the first time in years. By 2022, there was a positive recovery in TB diagnosis and treatment, helping to mitigate the pandemic's impact. However, most countries remain far from achieving End TB Strategy targets. Despite being preventable and curable, TB was the second leading cause of death from an infectious disease in 2022, following COVID-19, and caused nearly twice as many deaths as HIV/AIDS. Urgent action is needed to implement the commitments made at the 2023 UN high-level meeting on TB (World Health Organization, 2023).

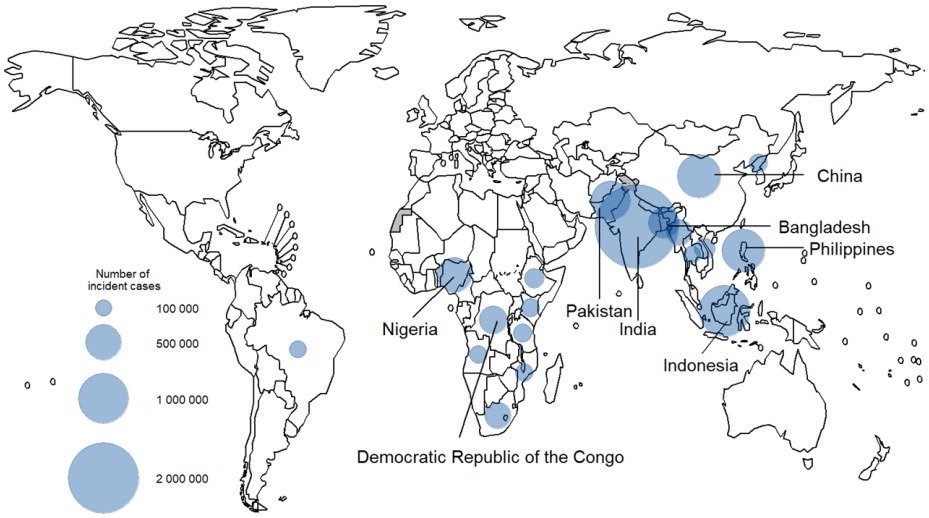

Figure 2. 1 The estimated number of incidence tuberculosis (TB) cases in 2022 for countries reporting at least 100,000 incidents.

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2.2.2 Prevalence of Tuberculosis in Malaysia

According to the 2022 WHO Global Tuberculosis Report, approximately 10.6 million new TB cases were diagnosed globally in 2021, with 1.6 million people dying from the disease. However, this figure represents only 70% of the actual TB cases due to underreporting and undiagnosed cases. The complexity of the TB epidemic is influenced by several factors, including the increasing incidence of HIV infections, antimicrobial resistance, migration from high TB-burden countries, the prevalence of non-communicable diseases, and patterns of international travel. In 2021, the largest proportion of TB cases, 45%, occurred in Southeast Asia. In Malaysia, TB cases have remained persistently high over the past 30 years, despite high cure rates being achievable with timely diagnosis and appropriate antibiotic treatment. Malaysia is classified as an intermediate TB burden country, with an estimated

incidence of 97 cases per 100,000 population (ranging from 79 to 106) in 2021. Containing TB has been a key priority in the strategic agenda of the Ministry of Health Malaysia (MOH) over the past few decades (Rashid et al., 2023). Malaysia reported 26,781 cases of tuberculosis in 2023, reflecting a 5.47% increase from the 25,391 cases recorded in 2022, according to the Ministry of Health (The Straits Times, 2024).

2.3 Pathogenesis of Tuberculosis

TB is transmitted through aerosol droplets containing *Mycobacterium tuberculosis*, which are expelled by individuals with active TB when they cough, sneeze, or speak. Once the bacteria are inhaled by a new host, they travel through the respiratory tract to the lungs. At this point, the host's innate immune system attempts to control the infection by internalizing the tubercle bacilli within alveolar macrophages. If the macrophages are unable to inhibit or destroy the bacilli, the bacteria multiply within the cells, are released, and subsequently phagocytosed by other macrophages, continuing the cycle. Lymphocytes are then recruited to the infection site, triggering a cell-mediated immune response, where an accumulation of immune cells attempts to contain the bacteria and prevent further replication. At this stage, the host remains asymptomatic, and the TB bacteria may either be completely eradicated or enter a latent phase within a granuloma. However, if the immune system is compromised, the infection can progress directly into active TB, presenting with clinical symptoms. After transmission of *Mycobacterium tuberculosis* to a new host, the bacilli enter the lungs and are engulfed by macrophages. Additional immune cells are then recruited to encase the infected macrophages, resulting in the formation of a granuloma, the defining feature of TB. In healthy individuals, the infection remains latent and controlled at this stage, although there is a risk of reactivation. When foamy macrophages undergo necrosis, they release lipid

content, leading to caseation, a cheese-like decay at the core of the granuloma, which weakens its structural integrity. As the granuloma progresses, the bacilli begin to escape from the macrophages into the caseous layer. During reactivation, *M. tuberculosis* multiplies, increasing the bacterial load to a critical level, eventually causing the granuloma to rupture and release the bacteria into the airways. The bacilli are then expelled as infectious aerosol droplets, which can infect new individuals, perpetuating the transmission cycle (Alsayed & Gunosewoyo, 2023).

2.4 Immune Response of Tuberculosis

2.4.1 Innate Immunity

Innate immunity plays a crucial role in defending the host against early *M. tuberculosis* (*Mtb*) infection, as seen in the ability of most *Mtb*-exposed individuals to spontaneously control the infection despite the delayed activation of acquired immunity. However, even a fully functioning adaptive immune system cannot limit *Mtb* growth in hosts lacking effective innate immune responses. Innate immune cells, as first responders, conduct immune surveillance through pattern recognition receptors (PRRs), which trigger cellular events such as phagocytosis and apoptosis to combat *Mtb*. Despite this, *Mtb*'s long history of co-evolution with humans enables it to evade these defense mechanisms, ensuring persistent infection. Furthermore, *Mtb* has developed sophisticated strategies to manipulate key components of the host's innate immune regulatory systems, such as intranuclear regulation, the ubiquitin system, and intrinsic immune elements, allowing it to evade immune clearance. This review outlines newly discovered mechanisms of *Mtb* immune evasion, including targeting cytosolic nucleic acid-sensing pathways, disrupting host cellular processes like membrane integrity,

autophagy, and cell death, and manipulating the molecular machinery of innate immunity to promote its intracellular survival.

2.4.2 Cell Mediated Immune Response

The immune response to *M. tuberculosis* is closely linked to the activity of Th1 cells, which produce mediators like IFN-γ, essential for activating infected macrophages. Naive *Mtb*-specific T cells proliferate in the draining lymph nodes following activation by dendritic cells (DCs), after which they migrate to the lungs, where they help control the infection. The importance of T cells in *Mtb* defense is highlighted by the impact of TNF-α blockers, which disrupt T cell proliferation and cytokine production, increasing the risk of infection or reactivation. The critical role of CD4+ T cells is further emphasized in HIV-positive patients, where their depletion leads to greater susceptibility to both primary infection and reactivation of latent TB. Persistent Th cell function is essential for maintaining a protective immune response during both acute and chronic *Mtb* infection. CD8+ T lymphocytes play a key role in the immune response against intracellular pathogens, including *M. tuberculosis*. The involvement of *Mtb*-specific CD8+ T cells is evidenced by their early presence in the airway lumen during infection. Their effector functions include: (1) lysing infected cells such as macrophages and dendritic cells, (2) producing IFN-γ, albeit in smaller quantities compared to CD4+ T cells, and (3) directly killing intracellular bacteria through the release of granzymes and perforins. Additionally, the long-term development and functionality of the CD8+ T cell response, in *Mtb* and other infections, is highly dependent on the presence and profile of memory CD4+ T cells, particularly those with a Th1 phenotype (Matucci et al., 2014).

2.4.3 Humoral Immune Response

Since 1921, the BCG vaccine has been used to prevent tuberculosis, but its effectiveness against pulmonary TB has varied significantly, with reported efficacy ranging from 0% to 80%. This inconsistency suggests that while Th1 cytokines and innate immunity contribute to protection, they are not sufficient on their own. Research on the role of B cells and antibody production in TB defense has been limited compared to the focus on macrophages and T cells. This is due to the belief that TB is primarily controlled by cell-mediated immunity, with T cells and macrophages playing key roles in attacking *Mycobacterium tuberculosis*, an intracellular pathogen. Traditionally, vaccines against intracellular pathogens like M. tb have focused on T-cell-based strategies. However, emerging evidence shows that B cells and antibodies may also play an important role, even in infections like TB that are typically thought to rely on cell-mediated immunity (Stewart et al., 2023).

Antibodies have been shown to help control intracellular pathogens like *Chlamydia trachomatis*, *Salmonella enterica*, and *Ehrlichia chaffeensis*, indicating that intracellular microbes might have extracellular phases, making them vulnerable to antibody-mediated responses. In viral infections, antibodies not only neutralize pathogens but also block viral replication in infected cells. Furthermore, B cells contribute to the immune response by influencing T cell memory and improving vaccine protection through antigen presentation and cytokine production. In TB, B cells form immune complexes that influence effector cells like dendritic cells and macrophages, potentially aiding in pathogen clearance, though the existence of specific neutralizing antibodies against *Mtb* remains unclear (Matucci et al., 2014). Understanding how B cells interact with cellular immunity may help improve strategies for controlling TB and enhance vaccine efficacy.

2.5 Immunodominant Tuberculosis Epitopes

Immunodominance is characterized by the immune response of an individual immunized with a specific protein. In this context, immunodominant epitopes can also be identified for B cells (Adorini, 1998). Although BCG remains the only available vaccine for TB, its limited ability to confer complete protection underscores the necessity of modifying BCG to express immunodominant antigens that could improve its protective efficacy (Arora et al., 2020). These epitopes are crucial for the development of effective vaccines and diagnostics against tuberculosis. Since only a select few immunodominant epitopes within an antigen are responsible for eliciting a strong immune response, the specificity and immune reaction to these epitopes can be readily identified and assessed. An epitope is a specific region on the surface of an antigen that is recognized by the immune system, particularly by B cells or T cells. In response to an epitope, the body produces antibodies that bind to it through the paratope. Among the various tuberculosis epitopes, the common immunodominant included are Antigen 85B (Ag85B), Alpha crystallin protein (Acr), and Resuscitation Promoting Factor E protein (RpfE).

2.5.1 Antigen 85B (Ag85B) Epitope

Several vaccines currently under investigation incorporate Ag85, a major immunodominant component of the *Mtb* complex, based on evidence that vaccination with these antigens provides protection against pulmonary *Mtb* infection in mice. Ag85B is typically secreted by replicating bacteria and is commonly detected in biological samples from TB patients. It is a virulence factor in tuberculosis, functions as a complex that binds to fibronectin (Fn) protein to facilitate the attachment and invasion of *Mtb* into human macrophages (Kuo et al., 2013).

It acts as a mycolyltransferase and consists of three proteins: Ag85A, Ag85B, and Ag85C. Of these, Ag85A and Ag85B are the predominant components, accounting for approximately 60% of the total proteins in the culture fluid of *Mtb* H37Rv, with Ag85B being more abundant than Ag85A. Ag85B can be detected in the culture fluid as early as three days. Therefore, targeting Ag85B as a biomarker is advantageous due to its abundance and activity (Karimah & Pambudi, 2020).

2.5.2 Alpha crystallin protein (Acr) Epitope

The membrane-associated heat-shock protein alpha crystallin (Acr/Rv2031c/HspX) of *Mtb* is thought to help the bacilli survive during the latent or dormant phase of infection. Its high production by dormant bacilli and its restriction to the *Mtb* complex make Acr a promising biomarker for latent TB infection. Acr is a potent inducer of both T and B cell responses and is considered one of the most immunogenic proteins of *Mtb*. Compared to active TB, individuals with LTBI have demonstrated a stronger T cell response to Acr, a response not affected by prior BCG vaccination. Reactivation of latent TB infection involves the renewed multiplication of dormant bacilli, potentially accompanied by changes in their expressed antigens. During the latent phase, *M. tuberculosis* notably over expresses a specific set of genes and proteins, with Acr being among the most prominent. Consequently, the immune response to *M. tuberculosis* can be viewed as dynamic, offering a foundation for the identification of novel biomarkers to track disease progression (Kumar et al., 2020).

2.5.3 Resuscitation Promoting Factor E protein (RpfE) Epitope

The risk of tuberculosis associated with DM depends on the prevalence of diabetes, making it a significant risk factor in populations with a high incidence of TB. Diabetes is known to

suppress the immune system, facilitating the activation of latent tuberculosis. In addition to its impact on immune responses, studies have shown that resuscitation promoting factors (RPFs) stimulate the growth of dormant mycobacteria (Verma et al., 2021). Moreover, RpfE has been identified by immune cells from individuals with latent tuberculosis infection, highlighting its significance in distinguishing between LTBI and active TB cases. Its role in promoting growth in mycobacterial cultures underscores its importance in understanding the pathogenicity of *M. tuberculosis* and in the development of effective diagnostic tools and vaccines (Gong et al., 2022).

2.6 Clinical Presentation of Tuberculosis

The symptoms of active TB disease vary depending on the area of the body where the TB bacteria are multiplying. In most cases, TB bacteria primarily grow in the lungs, which is known as pulmonary TB. When this occurs, individuals may experience a persistent cough lasting more than three weeks, chest pain, and the coughing up of blood or sputum from deep within the lungs. These are the hallmark symptoms of active TB in the respiratory system. However, TB can manifest with other general symptoms as well, including weakness, fatigue, weight loss, loss of appetite, chills, fever, and night sweats, which are common signs of the body fighting an infection (*Signs and Symptoms of Tuberculosis*, 2024).

Extrapulmonary TB occurs when the infection affects organs other than the lungs or in addition to the lungs, leading to symptoms specific to the affected area. For example, blood in the urine may suggest TB of the kidneys, while headaches or confusion could indicate TB meningitis. Back pain might point to TB in the spine, and hoarseness can signal TB of the larynx. Swollen glands may suggest TB in the lymph nodes, while swollen and painful joints

may be a sign of TB in the bones or cartilage (Centers for Disease Control and Prevention [CDC], 2024). When patients exhibit systemic symptoms and are at high risk for TB, extrapulmonary TB should be considered in their differential diagnosis to ensure appropriate evaluation and treatment.

2.7 Diagnosis and Treatment of Tuberculosis

2.7.1 Microbiological Diagnosis of TB

Pulmonary TB is often diagnosed based on clinical symptoms such as a persistent productive cough, hemoptysis, fever, weight loss, and a history of TB. Diagnosis is typically confirmed through chest X-ray, which may show alveolar infiltration, cavitation, lymphadenopathy, or pleural effusion. Sputum samples, bronchial or bronchioalveolar lavage, and tracheal aspirates are primary specimens for diagnosing pulmonary TB. Acid-fast bacilli (AFB) staining, commonly used in TB-endemic regions, detects *M. tuberculosis* but has limited sensitivity, particularly in the presence of nontuberculous mycobacteria. Culture methods, considered the gold standard, it takes about 2-6 weeks and are more sensitive than AFB staining. Commercial systems like the MGIT 960, VersaTREK, and MB/BacT Alert 3D can detect *Mtb* in approximately 10 days, improving diagnostic accuracy when multiple sputum samples are used (Gopalaswamy et al., 2020).

2.7.2 Molecular Diagnosis of TB

Molecular diagnostics have revolutionized tuberculosis detection by offering faster alternatives to traditional methods. Nucleic acid amplification tests (NAATs), such as the *Mycobacterium tuberculosis* Direct test, Xpert *Mtb*/RIF, and Amplicor, rapidly detect TB,

with results available in just a few hours. The Xpert *Mtb*/RIF system is particularly efficient, identifying both TB and rifampicin resistance in less than two hours, and the newer Xpert *Mtb*/RIF Ultra enhances sensitivity, especially for low-bacterial-load cases.

Additional molecular methods like loop-mediated isothermal amplification (LAMP) and cross-priming amplification (CPA) enable quicker detection with visual outputs, while the CE-IVD Genedrive offers portable testing. Tools like Anyplex II and EZplex kits are instrumental in detecting multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB. Line probe assays (LiPA) and whole genome sequencing (WGS) further aid in identifying drug resistance, though WGS remains costly for routine use in TB-endemic areas. Meanwhile, MALDI-TOF mass spectrometry is a cost-effective method for rapidly identifying mycobacteria in 1-2 hours (MacLean et al., 2020). Overall, these advancements are enhancing TB diagnosis and treatment efficiency.

2.7.3 Diagnosis of Latent TB

The diagnosis of latent TB infection (LTBI) relies on a few key methods, notably the tuberculin skin test (TST) and the Interferon-gamma release assay (IGRA). LTBI refers to individuals exposed to *Mycobacterium tuberculosis* (*Mtb*) without active disease symptoms. The TST involves injecting purified protein derivative (PPD) and measuring the skin induration after 48–72 hours. A positive result, typically an induration larger than 10mm, indicates previous exposure to *Mtb*; however, the test is non-specific and can yield false positives in individuals vaccinated with BCG. The IGRA, particularly the QuantiFERON-TB Gold In-tube Test (QFT-GIT) and T-SPOT.TB, uses *Mtb*-specific antigens to measure the immune response via interferon-gamma (IFNg) production. IGRA is preferred for BCG-

vaccinated individuals and those at low-to-moderate risk. An advanced version, QFT-Plus-IGRA, improves detection in cases involving CD8+ T-cell responses, but it remains costly for TB-endemic regions (Carranza et al., 2020).

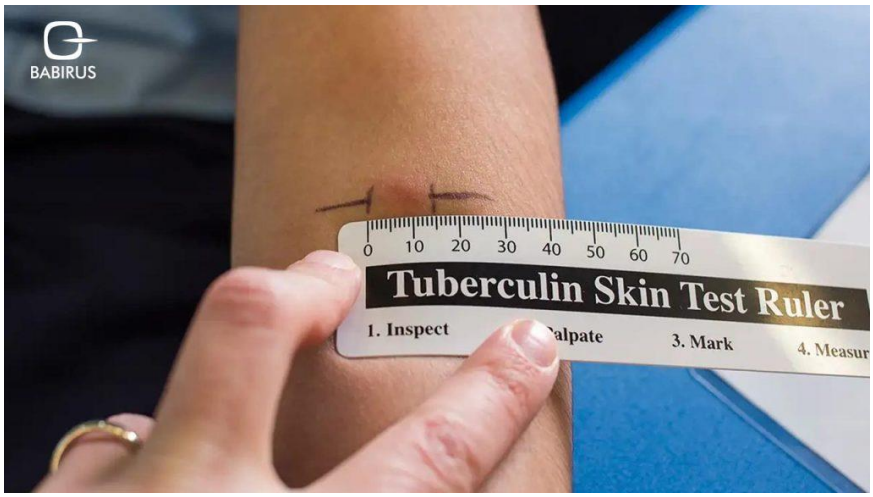

Figure 2. 2 shows the process of Tuberculin Skin Test (TST)

2.7.4 Drug Susceptibility of TB

For drug-resistant TB, accurate and rapid drug susceptibility testing (DST) is crucial. MDR-TB is resistant to first-line drugs like isoniazid (INH) and rifampicin (RIF), while XDR-TB also resists fluoroquinolones and at least one injectable second-line drug. Traditional DST takes 8-12 weeks using solid media, but automated liquid culture systems, like the Mycobacteria Growth Indicator Tube (MGIT), reduce the wait time to around 10 days. Additional methods, such as tyrosine kinase medium, microscopic observation of drug susceptibility (MODS), and FASTPlaque assays, provide faster results but often need confirmatory testing. Whole genome sequencing (WGS) is emerging as a tool for identifying drug-resistant mutations, showing good correlation with culture-based DST. Kits like the

Abbott RealTime *Mtb* RIF/INH Resistance and the Vere*Mtb* are known to be promising in detecting drug resistance, with reasonable sensitivity and specificity for RIF and INH resistance detection (Gopalaswamy et al., 2020).

2.7.5 Treatment of TB

The standard regimen for treating pulmonary tuberculosis (PTB) consists of an initial two-month phase of ethambutol, isoniazid, rifampicin, and pyrazinamide (2EHRZ), followed by four months of isoniazid and rifampicin (4HR), with daily dosing recommended throughout. For extrapulmonary tuberculosis (EPTB), specific regimens are advised: bone or joint tuberculosis should be treated with 2EHRZ followed by 4 to 7 months of HR, tuberculous meningitis with 2EHRZ followed by 10 months of HR, and other forms of EPTB with 2EHRZ/4HR. Corticosteroids are recommended for managing tuberculous meningitis and pericarditis. In cases of HIV-TB co-infection, antiretroviral therapy (ART) should be initiated within eight weeks of starting TB treatment, but in patients with a CD4 count below 50 cells/mm^3, ART should begin within the first two weeks. For those with TB meningitis, ART initiation should be delayed by two months. In patients using a protease inhibitor-based ART, rifabutin should replace rifampicin. Co-trimoxazole preventive therapy is recommended for HIV-TB patients with unknown or low CD4 counts (<200 cells/mm^3). Directly observed treatment (DOT) should be conducted for all TB patients to ensure adherence.

For adults with LTBI, the preferred treatment regimens are 3HR or 3HP unless contraindicated. For those unable to tolerate isoniazid (INH)-based regimens, 4R is recommended, while 6H or 9H is an option for those contraindicated for rifamycin-based regimens. HIV-positive adults may consider the 1HP regimen. In children with LTBI, the

preferred treatments are 4R for children older than 28 days and 3HP for those over 2 years, with 6H recommended for newborns under 28 days. Alternative regimens for children include 3HR, 6H, or 9H. For HIV-infected children, 6H is preferred for those under 2 years and for those aged 2 or older on antiretroviral therapy with rifamycin interactions (Malaysian Health Technology Assessment Section (MaHTAS), 2021).

2.8 Mucosal Immunity

Mucosal vaccination presents an appealing strategy for a primarily lung-based disease like TB because it stimulates both local and systemic immunity. Administering a vaccine through the respiratory mucosa can induce favorable immune responses within the local environment, where immunity generated by *Mycobacterium tuberculosis* may dominate. The mucosal immune system serves as the body's first line of defense against pathogens, consisting of inductive and effector sites. The inductive sites facilitate antigen uptake and the activation of naïve T and B cells, which then migrate to various mucosal effector sites. At the effector sites, secretory IgA (sIgA) is produced, initiating mucosal immunity. The mucosal surface in humans and animals is lined with epithelial and mucus-secreting cells that create a barrier, essential for separating the external environment from internal compartments. This barrier is critical in connecting host cells to their surroundings, and since mucosal epithelial cells are constantly exposed to external pathogens, they play a vital role in regulating immune responses. They are involved in microbial recognition, maintaining immune homeostasis, and interacting with antigen-presenting cells. The interaction with *Mycobacterium tuberculosis* is crucial for the bacterium's entry into the host, with epithelial M cells significantly contributing to this process. When the mucosal barrier is compromised,

epithelial cells can rapidly restore its integrity through programmed responses, maintaining the protective function of the mucosal surface.

Mucosal epithelial cells secrete various antimicrobial substances, including mucins, defensins, lysozyme, nitric oxide, and secretory IgA (sIgA), which collectively form a robust physical and antimicrobial defense. Mucins help maintain a balanced microbial flora, while defensins are effective against a broad range of pathogens, including *Mtb*. Nitric oxide and reactive nitrogen intermediates produced by alveolar macrophages further bolster defenses against *Mtb* infection. sIgA, the predominant antibody in mucosal tissues, plays a critical role in mucosal immunity by agglutinating pathogens, preventing their adhesion, and neutralizing toxins. This indicates that mucosal vaccination can significantly enhance sIgA production, suggesting that sIgA is vital in blocking *Mtb* entry at the mucosal surface and providing early protection against infection (Chai et al., 2019).