DEVELOPMENT OF TCR-LIKE SINGLE DOMAIN ANTIBODY BY INCORPORATING IMMUNOGLOBULIN ISOTYPES FOR LATENT TUBERCULOSIS DIAGNOSTICS AND THERAPEUTICS APPLICATION AGAINST HEAT SHOCK PROTEIN 16 KDA PEPTIDE PRESENTED BY HLA-A2

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by

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Five years have passed in the blink of an eye, and I have also spent a full five years in Malaysia, feeling a lot. Firstly, I would like to express my gratitude to my parents for their support and selfless love, which has been the greatest driving force behind my journey. Secondly, I would like to express my gratitude to my supervisor, Associate Professor Tye Gee Jun. He is very kind and friendly, who answered my questions on the project and led me into the field of antibody production and purification, making many meaningful explorations. Whether in work or life, Dr Tye has taken meticulous care of me. Finally, I would like to express my gratitude to Associate Professor Jiang Qichen from Nanjing Normal University, Jiangsu Institute of Freshwater Fisheries. Under the guidance of both Dr Tye and Jiang, I also explored the immune response caused by microplastics and the pharmacological properties of natural plant extract quercetin.

There are too many memories in Malaysia that I will never forget. The enthusiastic Malay waitress in the supermarket told me in standard and fluent Chinese where the product I wanted was placed. On the street, the Indian guy greeted me with extremely fluent Chinese to taste jackfruit on his stall and explained the knowledge of jackfruit. A young man who returned from studying in the UK to Malaysia for summer vacation gave us one durian after another to eat. We ate a lot, but he firmly refused to accept our money. There are many beautiful scenes like this. I am also about to graduate, this is an end, and I think it is a new beginning, I have

received job invitations from multiple Chinese universities. As a gratitude to Malaysia, I will provide opportunities for young people in Malaysia for a long time to come, especially undergraduate students from the Universiti Sains Malaysia, because they are young enough and also the hope of the country. I will provide them with job opportunities and bring them to China, because China has a sufficiently large market to support any wonderful ideas. More importantly, it is hoped that their experiences and growth will become a bond, connecting the exchanges between young people from China and Malaysia, and enabling them to find their own life goals and achieve their life ideals through mutual communication.

Writing here, my heart is excited, from the bottom of my heart, I am sincerely grateful to the country and people of Malaysia. Although I am leaving, as an old friend, a graduate from here, and my second hometown, I will definitely come back frequently to visit, hoping to contribute my humble efforts to the development and progress of Malaysia.

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LIST OF SYMBOLS

%	Percent
~	Approximately
+	Add
<	Less than
=	Equal to
>	More than
±	More or less
× g	Multiply by gravitational value
OD_{408nm}	Absorbance at wavelength 408 nm
°C	Celsius
cm	Centimetre
g	Gram
kDa	Kilodalton
L	Litre
M	Molar
mA	Milliampere
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
nm	Nanometre

™	Trademark
V	Volt
α	Alpha
β	Beta
γ	Gamma
μg	Microgram
μL	Microliter
μm	Micrometre

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzothaizoline-6-sulfonic acid)
ADCC	Antibody-dependent cell-mediated cytotoxicity
AEC-I	Alveolar epithelial cells of type I
AIDS	Acquired immunodeficiency syndrome
Ams	Alveolar macrophages
Amp	Ampicillin
APCs	Antigen presenting cells
APS	Ammonium persulfate
Atg5	Autophagy-related 5
BCG	Bacille Calmette-Guérin
BCR	B-cell receptor
BHK	Baby hamster kidney
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary
CO_2	Carbon dioxide
CGAS	Cyclic GMP-AMP Synthase
DC	Dendritic cell
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESX-1	ESAT-6 secretion system-1
ESAT-6	Early secretory antigenic target 6
FBS	Foetal bovine serum
Fc	Fragment crystallizable
FcR	Fc receptor
GM-CSF	Granulocyte macrophage colony stimulating factor
His	Histidine
HRP	Horseradish peroxidase
HSP	Heat shock protein
LAMP1	Lysosomal-associated membrane protein 1
LC3	Microtubule-associated protein 1 light chain 3
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin isotype A
IgD	Immunoglobulin isotype D
IgE	Immunoglobulin isotype E
IgG1	Immunoglobulin isotype G class 1
IgG2	Immunoglobulin isotype G class 2
IgG4	Immunoglobulin isotype G class 4
IgM	Immunoglobulin isotype M
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LTBI	Latent tuberculosis infection

MAPK	Mitogen-activated protein kinase
MCET	Mast cell extracellular trap
MCs	Mast cells
MDR-TB	Multidrug-resistant tuberculosis
MHC	Major histocompatibility complex
MTB	*Mycobacterium tuberculosis*
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
Ni-NTA	Nickel-nitriloacetic acid
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PknG	Protein kinase G
p62	Sequestosome 1
QC	Quality control
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
scFv	Single-chain fragment variable
TB	Tuberculosis

TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
Th	T-helper
Th1	Type 1 helper
Th17	Type 17 helper
Th2	Type 2 helper
TNF	Tumour necrosis factor
Treg	Regulatory T-cell
Tris	Hydroxymethyl
ULK	Unc-51-like kinase
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WHO	World health organization

LIST OF APPENDICES

PEMBANGUNAN ANTIBODI TUNGGAL SEPERTI TCR DENGAN MENGGABUNGKAN ISOTIP IMUNOGLOBULIN UNTUK APLIKASI DIAGNOSTIK DAN TERAPI TUBERKULOSIS LATEN TERHADAP PEPTIDA PROTEIN KEJUTAN PANAS 16 KDA YANG DIPERSEMBAHKAN OLEH HLA-A2

ABSTRAK

Protein kejutan haba 16 kDa (HSP 16 kDa) adalah penting untuk kemandirian *Mycobacterium tuberculosis* dalam fasa pendam. Oleh itu, molekul peptida-MHC HSP 16-kDa berpotensi sebagai sasaran diagnostik dan terapeutik untuk tuberkulosis pendam (LTB). Kajian ini bertujuan untuk menghasilkan format imunoglobulin yang berbeza pada antibodi domain tunggal (sDAb) mirip reseptor sell T (TCR) dan seterusnya menyiasat potensi diagnostik dan terapeutiknya dalam LTB, menggunakan sel model yang menpersembahkan peptida sasaran tersebut. sDAb mirip TCR yang telah dihasilkan sebelum ini yang boleh mengikat HSP 16 kDa telah digabungkan dengan bahagian krystal serpih (Fc) IgG1, IgG2, IgG4, IgA, IgD, IgE, dan IgM manusia melalui penyambung. Produk gabungan, sDAb-Fc, dihasilkan dalam sel HEK293-F dan kemudiannya ditulenkan. Potensi diagnostik dikaji melalui ELISA berasaskan sel yang menggunakan sel MCF-7 dengan peptida HSP 16 kDa. Sitotoksisiti sel yang diperantarai antibodi (ADCC) dari sel MCF-7 juga dijalankan untuk menyiasat potensi terapeutik. Akhirnya, sDAb mirip TCR-IgG1, IgG2, dan IgG4 berjaya dihasilkan secara sementara dalam sel HEK293 F dan ditulenkan menggunakan kromatografi protein A. Antibodi yang dihasilkan

diuji menggunakan ELISA berasaskan sel, yang menunjukkan pengikatan yang berkesan dari sDAb-IgG1, IgG2, dan IgG4 yang mirip TCR kepada peptida-MHC 16 kDa pada permukaan sel. Ujian ADCC juga menunjukkan bahawa antibodi secara efektif menghasilkan ADCC terhadap sel MCF-7 dengan bantuan peptida-MHC 16 kDa. Ini menunjukkan bahawa antibodi mirip TCR yang dibina mempunyai potensi yang baik untuk digunakan dalam diagnosis dan rawatan tuberkulosis terpendam.

DEVELOPMENT OF TCR-LIKE SINGLE DOMAIN ANTIBODY BY INCORPORATING IMMUNOGLOBULIN ISOTYPES FOR LATENT TUBERCULOSIS DIAGNOSTICS AND THERAPEUTICS APPLICATION AGAINST HEAT SHOCK PROTEIN 16 KDA PEPTIDE PRESENTED BY HLA-A2

ABSTRACT

Heat shock protein 16 kDa (HSP 16 kDa) is essential for the survival of latent *Mycobacterium tuberculosis*. Therefore, a peptide-MHC presentation of HSP 16 kDa could be a potential diagnostic and therapeutic target for latent tuberculosis (LTB). This study aimed to generate different immunoglobulin isotype pairings on a TCR-like single-domain antibody (sDAb) and subsequently investigate its diagnostic and therapeutic potential in LTB, utilizing a model cell presenting the target peptide. A previously generated TCR-like sDAb that can bind to HSP 16 kDa was first fused to a human IgG1, IgG2, IgG4, IgA, IgD, IgE, and IgM Fc-receptor via a linker. The fusion product, sDAb-Fc, was expressed in HEK293-F cells and subsequently purified. Its diagnostic potential was investigated via cell-based ELISA utilizing MCF-7 cells peptide-pulsed with HSP 16 kDa peptides. Antibody-dependent cell-mediated cytotoxicity (ADCC) of MCF-7 cells was also conducted to investigate its therapeutic potential. Finally, TCR-like sDAb-IgG1, IgG2, and IgG4 were successfully produced transiently in HEK293 F cells and purified using protein A chromatography. The generated antibodies were tested using cell-based ELISA, which demonstrated the effective binding of the TCR-like

sDAb-IgG1, IgG2, and IgG4 to the 16 kDa peptide-MHC on the cell surface. The ADCC assay also showed that the antibodies effectively mediated the ADCC of MCF-7 cells with the help of 16 kDa peptide-MHC. This indicates that the TCR-like antibody we constructed has good potential for application in the diagnosis and treatment of latent tuberculosis.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Mycobacterium tuberculosis (*MTB*) is the pathogen causing tuberculosis, which can invade all organs of the body, but it is the most common cause of pulmonary tuberculosis, which is the first cause of death from bacterial infectious diseases (Huang et al., 2023). The latent infection of *MTB* has no clinical symptoms of tuberculosis such as fever, cough, expectoration, hemoptysis or blood in sputum. There is currently no standard for the diagnosis of latent infection of *MTB*, the commonly used tuberculosis skin test (TST) and interferon-γ release assay (IGRA) to diagnose latent infection of *MTB* (Ying et al., 2023). Injecting pure protein derivative (PPD) of tuberculosis into the left forearm, TST is positive for the appearance of circular orange peel like bumps with a size of 7-8 mm on the local skin (Pahal & Sharma, 2022). The PPD antigen is susceptible to the influence of BCG vaccination. When the immune system of the body is disturbed by acute infectious diseases, the original response can be temporarily suppressed, and TST shows a negative reaction. If the body is complicated with malignant tumors, HIV infection, the TST response can be reduced or unresponsive (Charifa, Mangat, & Oakley, 2023). When the body is infected with *MTB*, sensitized T lymphocytes can produce IFN-γ, IGRA quantitatively detects IFN-γ level to determine whether the body is infected with *MTB*, but it cannot distinguish between active tuberculosis and

latent infection (Tebruegge et al., 2015). The World Health Organization has recommended multiple treatment options for latent tuberculosis infections. The drugs include : Isoniazid (INH), Rifapentin (RPT), Rifampicin (RIF) (Jereb, Goldberg, Powell, Villarino, & Lobue, 2011). However, these drugs often have a high risk of liver toxicity, and patients with liver dysfunction cannot use these drugs. At the same time, *MTB* is prone to develop drug resistance, making subsequent treatment more difficult (Jinbo, Lustik, West, & Kloetzel, 2017). Therefore, it is urgent to find effective diagnostic and treatment methods for latent *MTB* infections.

1.2 Problem statement

1.2.1 *MTB* against immune system

Some *MTB* can escape/evade the immune system and resides in the alveolus cells of the lungs and macrophages. This leads to the development of latent phase, the latent phase is difficult to diagnose. During latent phase, the humoral immunity is then rendered useless. T-cell responses makes it difficult for complete removal of *MTB*. When the immune system is compromised, it would exit the latent phase and reactivates TB. Therefore, there is a crucial need to diagnose latent TB and possibly a treatment for it.

1.2.2 Limitations of common antibodies

General antibodies lack the ability of immune surveillance in cellular immunity. Unable to detect *MTB* in cells.

1.2.3 Different immunoglobulin isotypes for TCR-like single domin antibody (sDAb)

Function of standard antibody isotypes have been determined. However, recombinant TCR-like sDAb fused with fragment crystallizable region (Fc region) has not been determined for diagnostics and therapeutics.

1.3 Rationale of study

TB is an infectious airborne disease caused by *MTB*. TB infection is divided into two major phases. During the initial active phase of TB infection, antibodies generated by the patients are capable of destroying the pathogen but some *MTB* will escape/evade the immune system and resides in the aveolar cells of the lungs. This leads to the latent phase whereby the humoral immunity is then rendered useless and would require the use of T-cells (cellular immunity) to mount a response(Makam & Matsa, 2021). Despite the capacity of T-cells to rid *MTB* infection, the latent phase is difficult to diagnose and the ability of *MTB* to continually evade T-cell responses makes it difficult for complete removal of pathogen. When the immune system is compromised, it would exit the latent phase and reactivates TB (Rahlwes, Dias, Campos, Alvarez-Arguedas, & Shiloh, 2023). Therefore, there is a crucial need to diagnose latent TB and possibly a treatment for it.

The 16 kDa antigen of heat shock protein (HSP) is expressed by *MTB* during the fixed period of bacterial hypoxia and nutrient deficiency, which is crucial for *MTB* survival during latent infections (C. Zhang, Yang, Zhao, Zhao, & Shi, 2018).

The frequency of HLA-A2 alleles is common worldwide (Ellis et al., 2000). In this study, the target peptide with binding ability to major histocompatibility complex (MHC) class 1 HLA-A2 was selected : GILTVSVAV, which has been proven to be derived from HSP 16 kDa (Dass, Norazmi, Dominguez, San Miguel, & Tye, 2018).

This project investigates the potential of a full format T-cell like receptor (TCR) antibody that could be utilised for biomedical research. The TCR-like antibody, which was previously generated targets a latent phase heatshock protein 16 kDa antigen of *MTB* presented by a common HLA-A2 molecule. Our main objective is to investigate the functional characteristics of TCR-like antibody that has been generated to be then converted into a full format antibody using human IgG1, IgG2, IgG4, IgA, IgD, IgE and IgM, immunoglobulin subtypes. We have generated a clone of TCR-like single domain antibody (sDAb) that have been shown to bind a heatshock protein 16kDa peptide (a fragment of protein that has been shown to be upregulated in latent TB) that is presented by the most common human HLA, HLA-A2. We hypothesised that by pairing the TCR-like antibody to immunoglobulin isotypes such as IgG1, IgG2, IgG4, IgA, IgD, IgE and IgM, we would be able to generate a full format antibody capable of diagnostics and/or therapeutics (**Figure 1.1**).

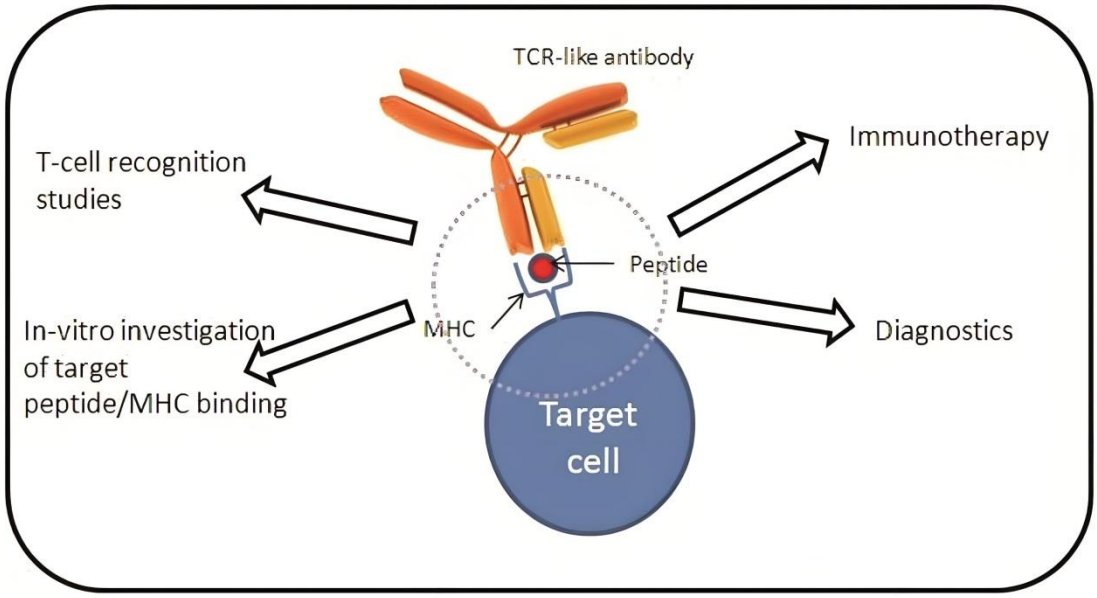

Figure 1.1 TCR-like single domain antibody for diagnostics/therapeutics purposes against TB antigens.

1.4 Research objectives

The main objectives of this project were as follow:

i. To investigate the optimal method of producing a full format TCR-like antibodies using different mammalian expression vectors from the initial sDAb binder.

ii. To optimize the production and purification of the full format TCR-like antibodies.

iii. To investigate the potential of the different full format TCR-like antibodies for diagnostics and therapeutic purposes using in-vitro cell models.

iv. To determine the effects of different Fc regions co-expressed with the binder.

CHAPTER 2

LITERATURE REVIEW

2.1 Interaction between the human's immune system and *MTB*

2.1.1 Overview of the state of TB

Tuberculosis has been associated with human beings for thousands of years and is an ancient infectious disease. *MTB* is the main pathogen of human tuberculosis. The human problem with *MTB* has never stopped from ancient times to modern society, and tuberculosis is still a serious health threat (Cambau & Drancourt, 2014). According to the Global tuberculosis Report 2022 issued by the World Health Organization (WHO), in 2022, there were 10.6 million new tuberculosis patients worldwide, and the global tuberculosis mortality rate will be 15%. Since 2019 to 2022, the number of deaths due to tuberculosis in the world has increased, changing the downward trend from 2005 to 2019. According to statistics, in 2022, a total of 1.6 million people worldwide died from tuberculosis; It was the second leading cause of death from infectious diseases after SARS-CoV-2 in 2020 (Bagcchi, 2023). In addition, the emergence of drug-resistant tuberculosis and multidrug-resistant tuberculosis (MDR-TB) and the lack of effective diagnostic methods for latent tuberculosis infection have brought many challenges to the prevention and treatment of tuberculosis (Saravanan et al., 2018). TB remains a significant public health challenge in Malaysia. The country has seen fluctuating TB rates, with an incidence rate of approximately 92 cases per 100,000 population in

recent years. This figure translates to around 30,000 to 35,000 new TB cases annually. Malaysia is considered an intermediate TB burden country, ranking 88th in the world(Mohamad Azranyi et al., 2024). TB is a leading cause of death among communicable, maternal, neonatal, and nutritional diseases in Malaysia (Qamruddin, Xavier, & Zahid, 2023). TB cases are more concentrated in urban areas due to higher population density, but rural areas are not exempt. Sabah and Sarawak, the two East Malaysian states, have reported higher TB rates compared to Peninsular Malaysia. TB cases found in Sabah, Malaysia account for 20% to 30% of the total population in the state(Goroh et al., 2020). While exact data on latent tuberculosis infection (LTBI) prevalence in Malaysia is limited, global estimates suggest that approximately one-quarter of the world's population is latently infected. Given Malaysia's TB burden, a significant proportion of the population is likely to have LTBI. High-risk groups in Malaysia include close contacts of active tuberculosis patients, medical staff due to occupational exposure, individuals with impaired immune systems such as those infected with HIV, migrant workers, and refugees who often come from tuberculosis-endemic areas and live in crowded environments(Wong & Lee, 2020).

2.1.2 Stages of *MTB* infection

MTB is a pathogen mainly transmitted through the air, and its infection process can be divided into four stages (**Figure 2.1**). In the first stage is infection, aerosols containing *MTB* are transmitted from infected individuals to healthy individuals. After entering the lungs, *MTB* is ingested into the cells by alveolar

macrophages (AMs) and dendritic cells (DCs) (I. Smith, 2003). Although AMs are considered effective barriers against pathogens, *MTB* has evolved various mechanisms to escape the host's immune response, such as inhibiting the secretion of inflammatory cytokines, blocking the production of reactive oxygen species and inhibiting antigen presentation, and inhibiting the acidification of phagosomes (Zhai, Wu, Zhang, Fu, & Liu, 2019). Through these mechanisms, *MTB* successfully resides in cells.

In the second stage is immune activation, the main characteristics of the stage of infection are the emergence of cellular immunity and the formation of granulomas. *MTB* escapes the killing of AMs, propagates in cells and causes cell damage, which attracts monocytes and other inflammatory cells in the blood to the infected site, but still cannot effectively kill bacteria. At 6 ~ 8 weeks after infection, DCs performing antigen presentation arrive in the lymph nodes, and T lymphocytes are activated and recruited (Shiloh, 2016). T lymphocytes that migrate to the infected site proliferate and form early granulomas with other immune cells. At this stage, bacterial growth and diffusion are restricted, but *MTB* can still be present in AMs Internal survival (Bold & Ernst, 2009).

The third stage of infection is the dormancy period. The formation of granulomas limits the spread of infection and regulates the environment in which *MTB* is exposed. The study recorded these environmental changes at the level of infected cells through the histochemical study of infected tissues of tuberculosis patients or by changing the growth conditions in the laboratory (Pagán &

Ramakrishnan, 2015). In addition to the stress caused by cytokine reactions, bacteria encounter low pH, hypoxia, and low nutritional stress. The low pH in macrophages is induced by the fusion of phagosomes containing *Mycobacterium* and lysosomal chambers. Hypoxia and low nutritional stress are caused by poor perfusion of the granuloma core, which is a mixture of dead and activated immune cells (Kiran, Podell, Chambers, & Basaraba, 2016). Under these unfavorable conditions, pathogenic mycobacteria can prevent their growth, trigger metabolic decline, and experience a dormant state. Pathogens can circulate in this state for several days or even decades (Esmail, Barry III, & Wilkinson, 2012). At this stage, there are no obvious signs of disease and the infection is under immune mediated control.

The final stage is the re-activation of latent *MTB*, the destruction of granulomatous structures, and the occurrence of pulmonary cavities and lung diseases, causing damage to the body (Gideon & Flynn, 2011). It is mainly caused by a decrease in host immunity due to genetic or environmental reasons. Mutations in host C-type lectins, cytokines, chemokines, and their specific receptors disrupt key signaling pathways involved in *MTB* immune responses. HIV infection causes damage to the host's immune function, especially $CD4^+$ T cells (target cells of HIV), which increases the host's susceptibility to *MTB* (Sasindran & Torrelles, 2011).

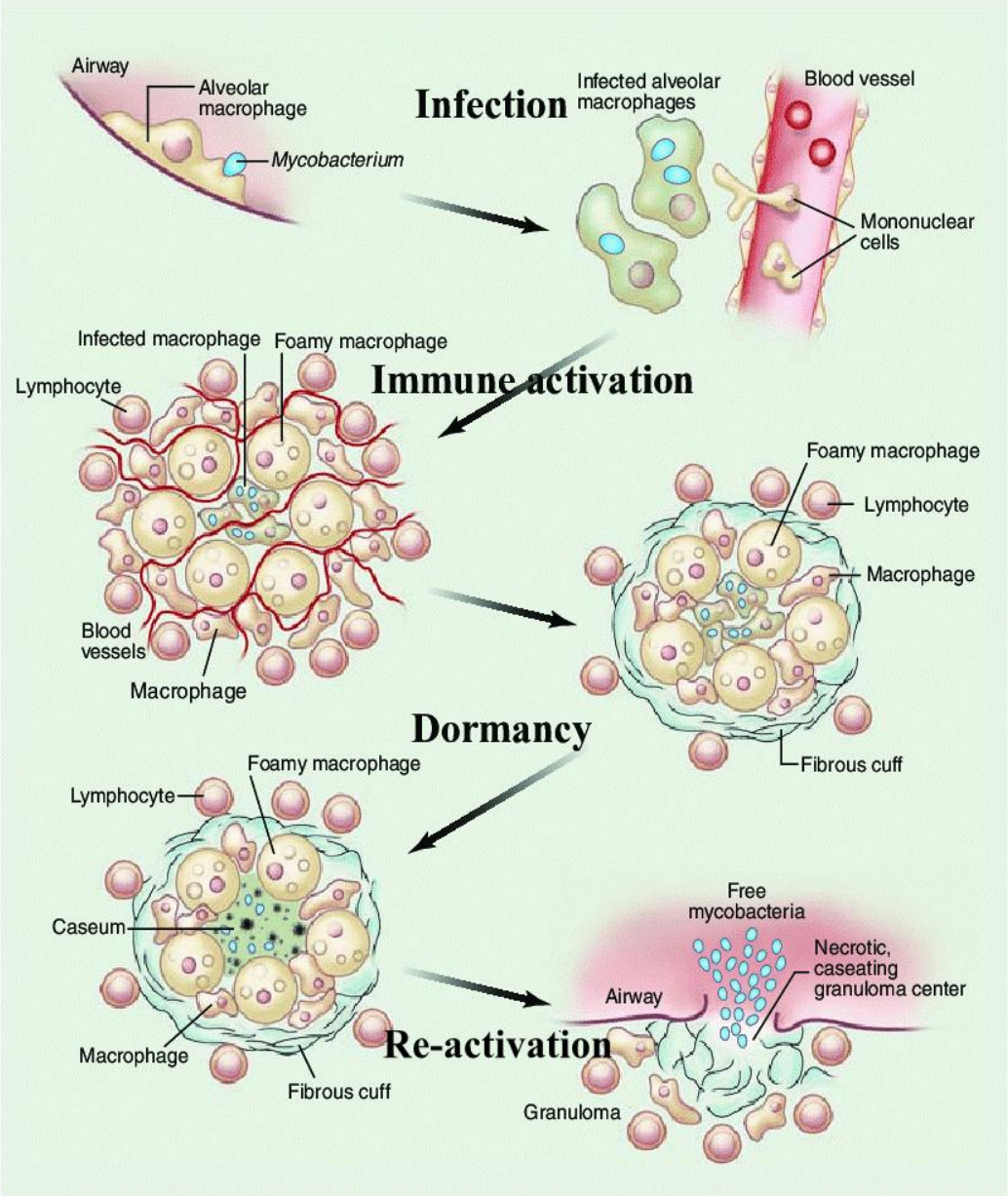

Figure 2.1 Life cycle of *MTB*. *MTB* infection follows a well-defined sequence of events, which can be divided into four stages, infection, immune activation, dormancy and re-activation (Sawyer, Grabowska, & Cortes, 2018).

2.2 Host immune response mechanism to *MTB*

The host's resistance to *MTB* infection requires a synergistic effect of innate and adaptive immunity. After *MTB* infection, the earliest contact between the host and the pathogen occurs in the innate immune response, and there is a lag in the host's adaptive immune response. However, more than 90% of infected individuals can successfully control *MTB* infection (Mayer-Barber & Barber, 2015). In addition studies have shown that mice with inherent immune signal deficits are significantly more susceptible to *MTB* (Thomas & Olive, 2023).

2.2.1 Airway mucosal barrier

To prevent the entry of harmful substances and eliminate foreign invaders, the surface of the body's airway is covered by a series of tightly adhered epithelial cells, which form the airway mucosal barrier (Ganesan, Comstock, & Sajjan, 2013). Airway mucus is a viscoelastic gel, which contains a large number of highly glycosylated mucins, such as defensins, immunoglobulins, lysozyme and cytokines. Defensins are the most important bactericidal component in mucus, which can kill various Gram positive and Gram negative bacteria (C. He et al., 2023). Immunoglobulin A (IgA) can prevent pathogen adhesion and neutralize its toxins, and IgA deficient mice have an increased susceptibility to Bacille Calmette-Guérin vaccine (BCG) compared to normal mice. Intranasal administration of IgA targeting *Mycobacterium* antigens significantly reduced the burden of *MTB* in the lungs of mice, and its effect was better when combined with interferon γ (Moliva, Turner, & Torrelles, 2017).

When inhaled into the lungs, *MTB* moves through the trachea, bronchus, and bronchioles to reach the alveoli. The respiratory mucosa, which lines the airway, consists of a layer of airway epithelial cells (AECs). These cells create a tight barrier to prevent *MTB* invasion and possess numerous receptors to detect the pathogen. AECs regulate the composition of airway surface liquid (ASL), which includes mucus, antimicrobial peptides, antibodies, and cytokines/chemokines. The lamina propria supports the epithelium and houses immune cells like macrophages and mucosal-associated invariant T cells (MAITs) that respond to infections. *MTB* eventually reaches the alveoli, which are encircled by capillaries to facilitate gas exchange. The alveolus structure is formed by type I epithelial cells, with type II epithelial cells commonly found at cell junctions. Type II cells secrete various antimicrobial substances, including pulmonary surfactant. AMs and DCs serve as the primary resident defenders in the alveolus, acting as effective phagocytes with extensive antimicrobial capabilities. Additionally, neutrophils and NK cells are recruited from nearby capillaries to strengthen the host defense (**Figure 2.2**)(Graf, Trautmann-Rodriguez, Sabnis, Kloxin, & Fromen, 2023). The Alveolar epithelial cells of type I (AEC-I) and type II (AEC-II) alveolar epithelial cells. AEC-II accounts for approximately 7% of the total alveolar surface and is capable of secreting cytokines and chemokines, as well as expressing main histocompatibility complex II (MHC II) on its cell surface (Chuquimia Flores, 2013). Target peptides can be presented to $CD4^+$ T cells by AEC-II (Chuquimia et al., 2012). In addition, AEC-II can secrete a mixture of surface active proteins, where Surfactant protein A

(SP-A) and Surfactant protein D (SP-D) are hydrophilic and belong to the lectin family. They are highly efficient innate immune molecules involved in virus neutralization, bacterial, fungal, apoptotic, and necrotic cell clearance (Carreto-Binaghi, Aliouat, & Taylor, 2016). In addition, alveolar epithelial cells also possess multiple pattern recognition receptors that can recognize *MTB* and induce host pro-inflammatory immune responses (Stamm, Collins, & Shiloh, 2015).

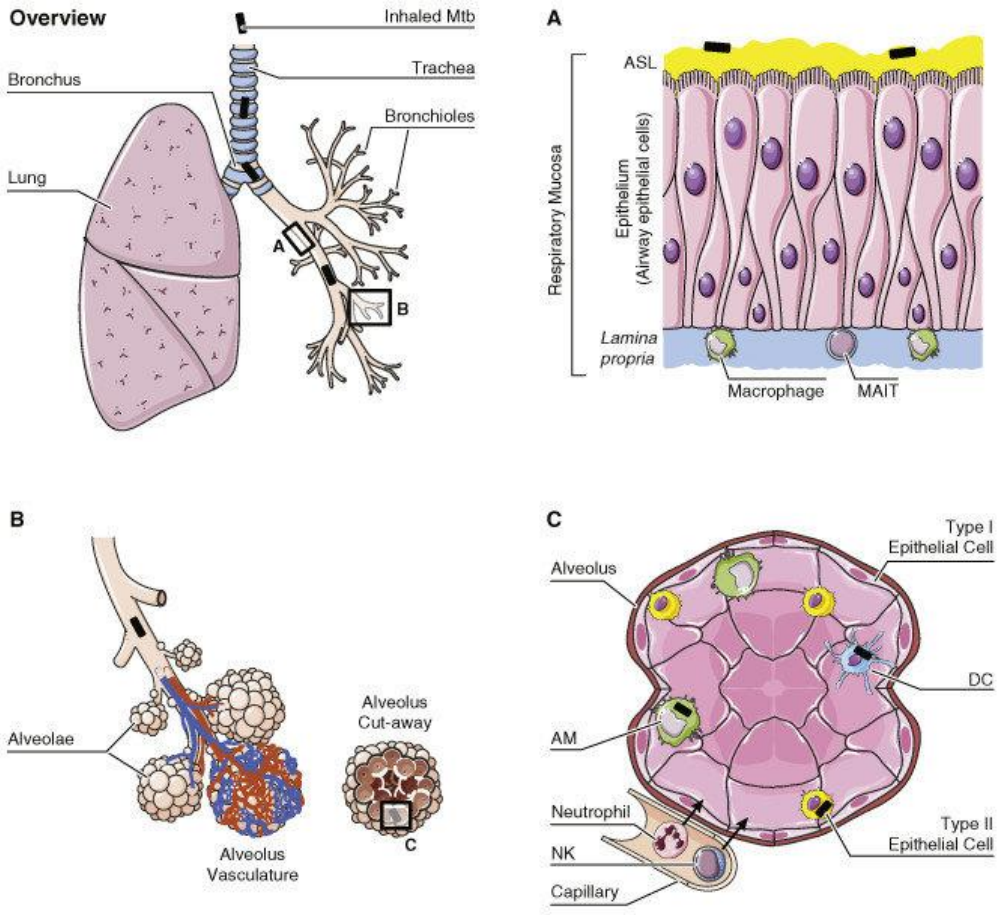

Figure 2.2 Cells involved in the human innate immune response to tuberculosis. Upon inhalation into the lung, *MTB* (black rod) travels along the trachea, bronchus and bronchioles to the alveoli. Lining the airway is the respiratory mucosa (A). *MTB* eventually reach the alveolae (B), which are surrounded by a network of capillaries to facilitate gas exchange. The alveolus (C) have a range of intrinsic anti-microbial capacities (Lerner, Borel, & Gutierrez, 2015).

2.2.2 Macrophages response to *MTB*

Macrophages are the first immune cells encountered by *MTB* during infection and are also the main host of *MTB*. Macrophages use pattern recognition receptors on the cell surface, such as Toll like receptor (TLR) Nod-like receptor (NLR) and C-type lectin receptor (CLR) to quickly recognize pathogen related molecular patterns (PAMPs) of *MTB*, such as glycolipids, lipoproteins, and engulf them into phagosomes, killing *MTB* through fusion with lysosomes and subsequent acidification of phagocytic lysosomes (C. H. Liu, Liu, & Ge, 2017). However, *MTB* has adapted to this bactericidal method of macrophages and adopted various strategies to inhibit phagocytic maturation and phagocytic lysosomal generation. In addition to interfering with the host, *MTB* can also resist the phagocytic environment of nitrification, oxidation, functional hypoxia, and carbohydrate deficiency through the expression of stress adaptive genes (Maphasa, Meyer, & Dube, 2021). In addition, macrophages infected with *MTB* express a series of inflammatory cytokines, chemokines, and antimicrobial agents to control the infection of *Mycobacterium* (Domingo-Gonzalez, Prince, Cooper, & Khader, 2016).

The *MTB* secretion system ESAT-6 secretion system-1 (ESX-1) is known for its ability to disrupt the integrity of phagosomes and prevent their maturation. ESX-1's destruction of phagosomes exposes bacterial DNA to the cytoplasmic DNA sensing pathway of macrophages, activating type I interferon (IFN) signaling, leading to *MTB* targeting autophagosomes for subsequent killing Cyclic GMP-AMP Synthase (cGAS) is necessary for macrophages to control *MTB* infection, and

compared to normal mice, cGAS deficient mice have an increased mortality rate after infection with *MTB* (Osman et al., 2022). However, excessive Type I IFN signaling can lead to host failure in controlling *MTB* infection and worsening of the disease. Autophagy can promote the maturation of phagosomes and enhance their ability to kill *Mycobacterium* (Ni Cheallaigh, Keane, Lavelle, Hope, & Harris, 2011). Studies have found that antimicrobial peptides can exert bactericidal functions by activating the transcription of host autophagy related genes Beclin-1 and autophagy-related 5 (Atg5). However, interestingly, the absence of autophagy related genes Unc-51-like kinase 1 (ULKl), Unc-51-like kinase 2 (ULK2), or Sequestosome 1 (p62) has no significant impact on controlling *MTB* infection.

Intracellular *MTB* has developed various strategies to overcome the antibacterial defense of macrophages (**Figure 2.3**). *MTB* inhibits the recruitment of reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase to phagosomes containing pathogens, thereby promoting the intracellular growth of *MTB* (Koster et al., 2017). *MTB* inhibits autophagy by inducing miR-33 expression and downregulating the expression of various autophagy proteins such as Atg5, Atg12, microtubule-associated protein 1 light chain 3 (LC3), and lysosomal-associated membrane protein 1 (LAMP1) (Ouimet et al., 2016). *MTB* also targets UVRAG by expressing miR-125a with the Beclin-1 complex, thereby inhibiting autophagy in macrophages and allowing cells to live *MTB* during this process (J. K. Kim et al., 2017). More and more discoveries explain why macrophages are the main host of *MTB*.

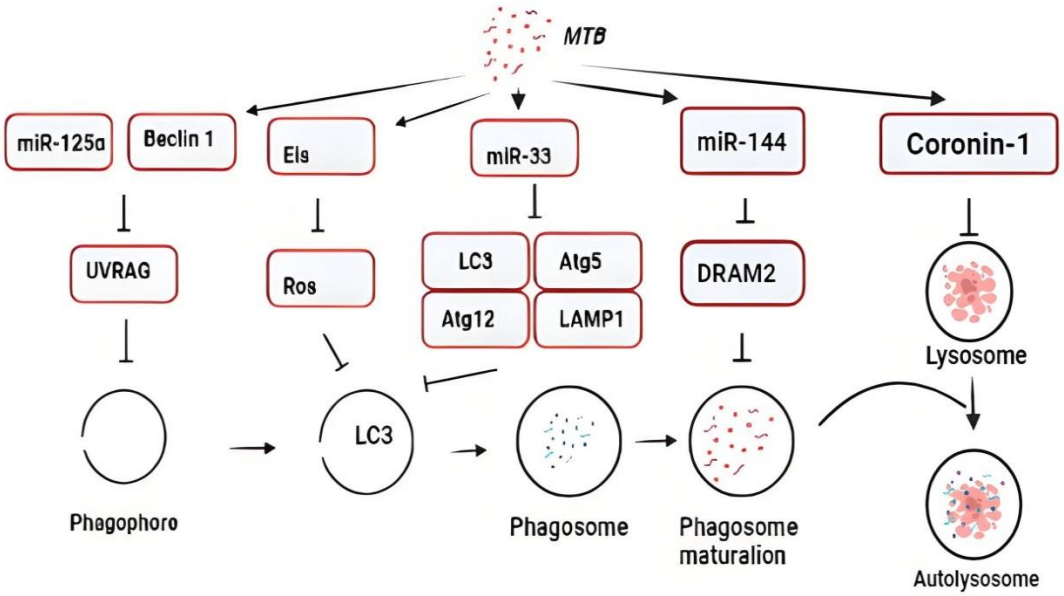

Figure 2.3 The evasion of autophagy by intracellular *MTB*. Intracellular *MTB* have been shown to employ various strategies to evade autophagy in macrophages. Figure adapted from Meyer 2021 et al (Meyer, Maphasa, & Dube, 2021).

2.2.3 Dendritic cells response to *MTB*

Dendritic cells (DCs) have the function of stimulating initial T cells. Most of the dendritic cells in the body are in an immature state and have strong antigen endocytosis ability, which can present extremely low concentrations of antigens (Reis e Sousa et al., 1997). DCs fortify the immune system's cellular defenses against *mycobacterium* infection (Mihret, 2012), at the beginning of the inflammatory response against *MTB*, DCs develop and move into lymphoid organs, where they secrete immune-regulating cytokines like IL-12 to activate T cells (Ouimet et al., 2016). DC is responsible for secreting polarized cytokines and

inducing $CD4^+$ T cells to differentiate into various subtypes (Helper T cells, Thl, Th2, Th17, and regulatory T cells) (Nam et al., 2021). After exposure to intracellular pathogens, DCs cells secrete IL-12, which differentiates $CD4^+$ T cells into Th1 cells. Subsequently, Th1 cells secrete IFN-γ, causing DCs to further produce IL-12 and induce the production of more Th1 cells. Correspondingly, DCs utilize IL-6 to induce the production of Th2 cells, which secrete IL-4 to further produce more Th2 cells (Vacaflores, Chapman, Harty, Richer, & Houtman, 2016). *MTB* protein PE27 activates DCs through mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways, inducing Th1 type immune response (W. S. Kim et al., 2016). The *MTB* protein PE27 activates DCs through MAPK and NF-κB signaling pathways, inducing Th1 type immune response. *MTB* specific Th1 type immune response is mediated by $CD11b^+$ DCs, and it is also negatively regulated by IL-10 produced by $CD103^+$ DCs (Choi et al., 2018). In summary, the initiation of adaptive immune response requires the participation of DCs, and enhancing the interaction between DCs and T cells may enhance the host's ability to resist *MTB*. The addition of exogenous CD40 agonists during *MTB* infection enhances the function of DCs and promotes *MTB* specific $CD4^+$ T cell response (Enriquez et al., 2022). Therefore, a better understanding of the function of DCs is conducive to the development of treatment methods for tuberculosis.

2.2.4 NK cells response to *MTB*

Natural killer (NK) cells are granular innate lymphocytes that express CD16 and CD56, with strong cytolytic ability (Ferlazzo et al., 2004). The various

components of *MTB* cell wall can directly bind to the natural cytotoxic receptor NKp44 on NK cells. Research has found that NK cells isolated from healthy donors can lyse *MTB* infected monocytes and reduce intracellular *MTB* growth (Abebe, 2021). For *MTB*, the direct killing mechanism of NK cells includes cytoplasmic granules containing perforin, granulolysin, and granzyme, as well as death receptors that can initiate cell apoptosis (**Figure 2.4**). In addition, NK cells can indirectly induce immune responses by activating macrophages and various signaling pathways, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Allen et al., 2015). In addition, NK cells inhibit intracellular growth by producing IFN-γ and IL-22 to enhance the fusion of phagocytic lysosomes. NK cells can also promote the proliferation of T cells by producing CD54, tumor necrosis factor α (TNF-α), Granulocyte macrophage colony stimulating factor (GM-CSF), and IL-12 (Abel, Yang, Thakar, & Malarkannan, 2018). In addition to the early innate immune function, NK cells are also present in pulmonary granulomas of *MTB* infected patients (Carabalí-Isajar et al., 2023). A better understanding of the role of NK cells in anti mycobacterial immunity and its molecular mechanism can open up new possibilities for NK cell based tuberculosis treatment strategies.

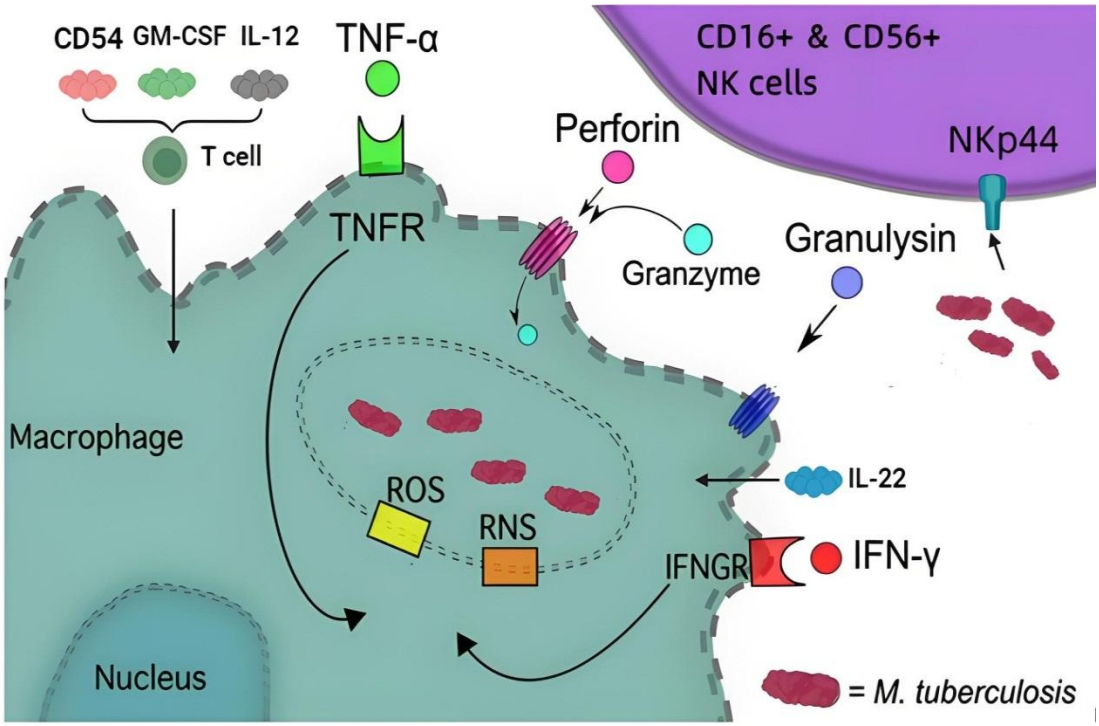

Figure 2.4 NK cells inhibit *MTB* growth via IFN-γ, TNF-α, perforin and granulysin, and granzyme. Figure adapted from Allen 2015 et al (Allen et al., 2015).

2.2.5 Other innate immune cells response to *MTB*

Mast cells (MCs) proliferate from blood stem cells, move to different tissues, and mature under the influence of local microenvironment (Valent et al., 2020). MCs promote immune responses through various mechanisms, such as bacterial recognition, recruitment of immune cells to infection sites, release of inflammatory mediators, and direct killing bacteria. However, its main function is to coordinate other immune cells against pathogen infection (Garcia-Rodriguez, Goenka, Alonso-Rasgado, Hernández-Pando, & Bulfone-Paus, 2017).

Since little is known about the contribution of MCs to TB pathogenesis, it is summarized the strategies used by MCs in bacterial defense, as well as the potential and reported interactions occurring between *MTB* and MCs (**Figure 2.5**), Shortly

after infection with *MTB* in guinea pigs, a significant increase in MCs was detected in the lungs (Ratnam, Ratnam, Puri, & Chandrasekhar, 1977). A subsequent study showed that after 20 days of *MTB* exposure, MCs in mice's lungs increased by around 23% (Carlos et al., 2007).

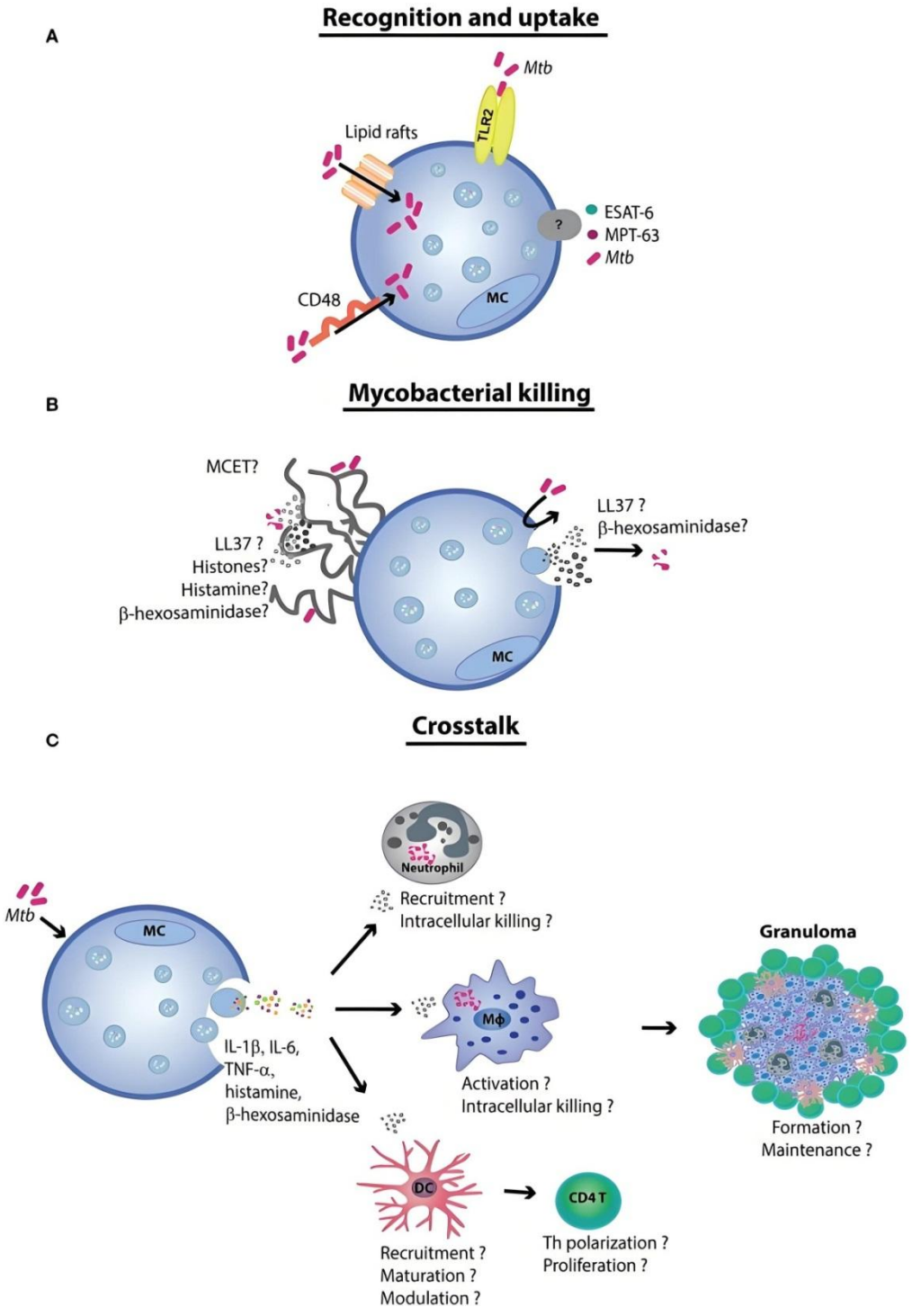

Figure 2.5 The role of mast cells (MCs) in tuberculosis. (A) MCs recognize *MTB* via the TLR2 and CD48 receptors. The latter also contributes to *MTB* uptake. (B) *MTB* and the mycobacterial antigens early secretory antigenic target 6 (ESAT-6) and MPT-63 induce MC degranulation and cytokine release. It is likely that *MTB* exposure induces antimicrobial peptide secretion and mast cell extracellular trap (MCET) formation. (C) Finally, the MCs crosstalk with other immune cells [e.g., neutrophils, dendritic cells (DCs), and macrophages (Mφ)] contribute to antimycobacterial immunity. MCs seem to play a role in orchestrating tuberculosis granuloma formation and maintenance (Garcia-Rodriguez et al., 2017).

2.3 Host adaptive immune mechanism against *MTB*

The control of *MTB* infection cannot be achieved without adaptive immune response. The secretion of cytokines and the direct antibacterial effect of antigen-specific T cells are key features of the host's adaptive immune response to *MTB* infection. The gradual loss of $CD4^+$ T cells in HIV patients leads to an increased susceptibility of the host to *MTB* (Sia & Rengarajan, 2019).

2.3.1 $CD4^+$ T cells

$CD4^+$ T cells have significant immune effects during *MTB* infection (Serbina, Lazarevic, & Flynn, 2001). HIV infection leads to down-regulation of $CD4^+$ T cells, leading to progressive primary TB infection, reactivation of LTBI, and increased susceptibility to reinfection(Sia & Rengarajan, 2019). In 2020, there were 208,000 TB deaths among people living with HIV (PLHIV), a significant decrease from the 678,000 deaths recorded in 2000 (Organization, 2023). The prevalence of TB-HIV coinfection in Malaysia was 12.6 % in 2010 and had decreased to 5.9 % by 2019. Despite the overall increase in TB prevalence in Malaysia since 2000, the prevalence of TB-HIV coinfection has remained below 6 % since 2014 (Graf et al.,

2023). The risk of patients with diabetes and chronic diseases also suffering from TB is significantly higher than that of normal people (Young, Critchley, Johnstone, & Unwin, 2009). In Kelantan, Malaysia, 26 ~ 29 % of TB patients were identified with diabetes mellitus (DM) annually. Although the incidence of TB has been decreasing, the number of diabetics among TB cases significantly and progressively increased over the five-year period from 2012 to 2016 (S. R. Ahmad, Yaacob, Jaeb, Hussin, & Mohammad, 2020). More and more evidence shows that $CD4^+$ T cells play a key role in controlling tuberculosis infection (Sakai et al., 2016).

$CD4^+$ T cells have many subpopulations, such as Thl, Th2, Th17, and regulatory T cells (Treg) (**Figure 2.6**). The *MTB* specific $CD4^+$ T cell immune protective response is usually caused by Th1 cells, which recruit innate immune cells and promote their antibacterial activity by secreting IFN-γ and TNF-α. These Th1 cells activate effectors in macrophages to kill *MTB*, and they are essential for the protective immunity of tuberculosis (Sakai, Mayer-Barber, & Barber, 2014).

Th17 cells are also involved in immune protection against *MTB* infection, and these cells recruit and activate neutrophils by producing IL-17. Thl7 cells are involved in immune protection against *MTB* in the early stages, and they can recruit monocytes and Th1 lymphocytes to the site of granuloma formation. However, unrestricted IL-17 stimulation can cause excessive inflammation and lead to damage to the body. Th2 cells can secrete IL-4 and IL-5 during *MTB* infection, which can promote the maturation of B lymphocytes. However, due to the reverse regulation of Th2 cells by Thl cells, they may weaken their immune protection against *MTB*

infection (Torrado & Cooper, 2010). Tregs play a crucial role in establishing and maintaining peripheral immune tolerance. At present, there is sufficient evidence to suggest that defects in the quantity or function of Treg cells can lead to autoimmune diseases, allergies, and transplant rejection. However, excessive Treg cells can inhibit the body's immune response to pathogens and tumors (Goswami et al., 2022). In vivo, the specific immune response and growth of tuberculosis bacteria can be inhibited by $CD4^+$ Treg cells in alveoli and pleural effusion. Treg cells can also inhibit peripheral effector T cell function, mainly by releasing inhibitory cytokines such as IL-10 and TGF-β, alternatively, its function can be achieved through cellular contact mechanisms (Pandiyan, Zheng, Ishihara, Reed, & Lenardo, 2007). Meanwhile, Treg also expresses unique chemokines that can mediate its migration to the lesion site and downregulate effector T cell function. Therefore, Treg is involved in the immune defense process of *MTB* intracellular infection, but it is inhibitory on the body's anti-tuberculosis immunity to maintain a balance of anti-tuberculosis immunity. It has been confirmed in mouse disease models that regulating the number or function of Treg cells can largely suppress pathological processes including transplantation, autoimmune diseases, and tumors (Schlöder, Shahneh, Schneider, & Wieschendorf, 2022). In chronic infections such as tuberculosis, malaria, HIV, and HCV, Treg cell function is overactivated, inhibiting the body's immune response to specific antigens. This may be one of the reasons for the persistence of infection and the inability to completely eliminate pathogens (Schlöder et al., 2022).

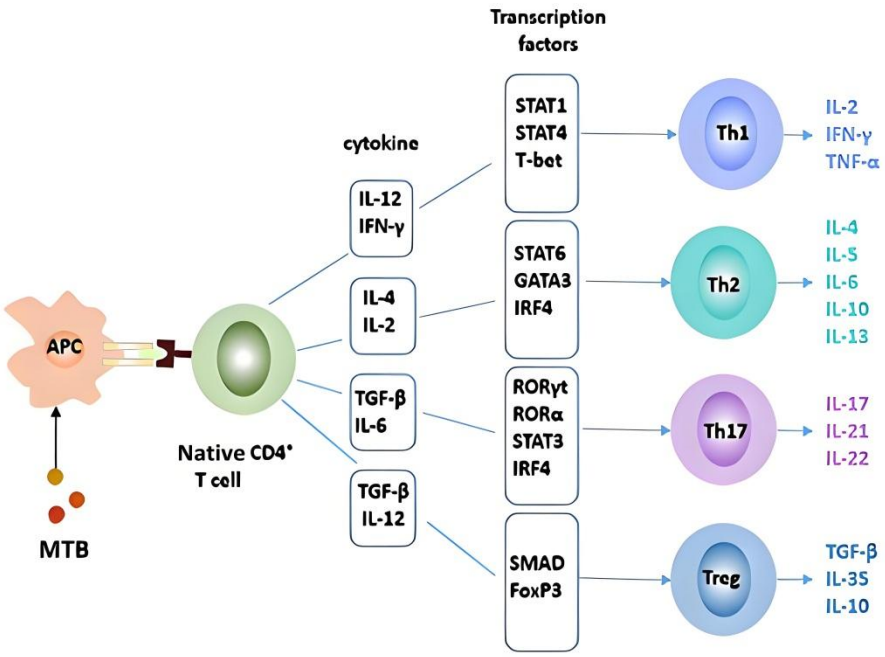

Figure 2.6 Schematic representation of the immune cells involved in *MTB* infection. Distinct types of T helper (Th) cells and regulatory T cells (Treg) are present at the site of *MTB* infection (Cantini et al., 2017).

2.3.2 $CD8^+$ T cells

Although initially believed to be less important than $CD4^+$ T cells in the immune response of *MTB*, it is now recognized that they play a crucial but complex role. $CD8^+$ T cells can recognize *MTB* specific antigens presented by classical (MHC Ia: HLA-A, HLA-B, HLA-C) and non classical (MHCIb: HLA-E, CD1) (van Meijgaarden et al., 2015). *MTB* antigens can be processed through transporter associated with antigen processing (TAP). $CD8^+$ T cells can produce cytokines with critical functions, such as IL2, IFN-γ and TNF during *MTB* infection (Woodworth & Behar, 2006). Importantly, $CD8^+$ T cells possess cytolytic function, which can induce cell apoptosis through particle mediated functions (through perforin, granzyme, and granolylysin) or Fas-Fas ligand interactions, thereby killing cells infected with *MTB* (**Figure 2.7**). $CD8^+$ T cells can lyse macrophages infected with