ACTIVATION OF HUMAN DENDRITIC CELLS BY LIPOSOMES DERIVED FROM TOTAL LIPID OF *Mycobacterium smegmatis*

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UNIVERSITI SAINS MALAYSIA

2023

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by

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

SEPTEMBER 2023

ACKNOWLEDGEMENT

In the name of Allah, the Most Generous and the Most Merciful.

I would like to express my gratitude to Allah SWT for granting me the opportunity, strength, and motivation in the completion of this thesis entitled 'Activation of human dendritic cells by liposomes derived from the total lipid of *Mycobacterium smegmatis'*. A special gratitude to my supervisor, Dr. Ramlah Kadir, for the guidance to conduct this research project under the Fundamental Research Grant Scheme (FRGS) (FRGS/1/2018/SKK08/USM/03/1). In addition, special thanks to my co-supervisors, Associate Prof. Dr. Siti Suraiya Md Noor, Prof. Dr. Armando Acosta, and Dr. Rohimah Mohamud.

I am beyond grateful for the endless help from the staff of the Department of Immunology, Mr. Jamaruddin Mat Asan and Mr. Azlan Mat Nasir, SEM PPSK and confocal PPSG staff, Mrs. Wan Norhasikin Wan Marizam and Mrs. Siti Fadilah Abdullah and to the medical doctors in 7 Utara and 2 Delima Hospital USM. To my colleagues, Dr. Suhana Ahmad, Dr. Nur Diyana Mohd Shukri, Fatmawati Lambuk, Dr. Lidawani Lambuk, and Aina Akmal Mohd Noor, thank you for the friendship.

Lastly, my greatest and everlasting gratitude is dedicated to my beloved Daddy, Ahmad Suhaimi Mahmud, Mom, Rohayati Abd Razak, and siblings, Ahmad Qhairul Nizam Ahmad Suhaimi and Ahmad Faizuddin Najmi Ahmad Suhaimi, for their constant prayers, sacrifices, and spiritual support. To Mom and my late Daddy, thank you, for encouraging me this whole time. This achievement is for both of you.

Thank you again to all who have contributed directly and indirectly to this study.

Nurfatihah Azlyna binti Ahmad Suhaimi

P-UM0011/19(R)

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Abbreviations	Definition
APCs	Antigen presenting cells
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
cDCs	Conventional dendritic cells
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
COVID-19	Coronavirus disease 2019
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DMT	Dimethyldioctadecylammonium
DT	Diphtheria toxoid
EBA	Early bactericidal activity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
E. coli	*Escherichia coli*
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen - DR isotype

HMDS	Hexamethyldisilane
IFN-γ	Interferon gamma
IFNGR	Interferon gamma receptor
IL	Interleukin
ILT1	Immunoglobulin-like transcript 1
INH	Isoniazid
Lin-1	Lineage-specific marker
LNs	Lymph node
LPS	Lipopolysaccharide
LTBI	Latent TB infection
MARCO	Macrophage receptor with collagenous structure
MCG	Multinucleated giant cells
MCO	Movement control order
mDCs	Myeloid dendritic cells
MDR-TB	Multidrug-resistant tuberculosis
MHC	Major histocompatibility complex
MLVs	Multilamellar vesicle
MMG	Monomycoloyl glycerol
moDCs	Monocytes-derived DCs
MPLA	Monophosphorylate lipid A
MPS	Mononuclear phagocyte system
MS	Multiple sclerosis
M. smegmatis	*Mycobacterium smegmatis*
Mtb	*Mycobacterium tuberculosis*
NA	Nutrient agar

NB	Nutrient broth
NFκ-B	Nuclear factor kappa B
NK	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDCs	Plasmacytoid dendritic cells
PEG	Polyethylene glycol
PPD	Purified protein derivative
PRRs	Pattern recognition receptors
REV	Reverse-phase evaporation vesicles
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SLE	Systemic lupus erythematosus
SSC	Side scatter
SUVs	Small unilamellar vesicles
T1D	Type I diabetes mellitus+B48
TB	Tuberculosis
TDB	Trehalose-6,6'-dibehenate
Th	T helper
TLRs	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
tol-DCs	Tolerogenic DCs
Tregs	Regulatory T cells
TST	Tuberculin skin test

WHO	Worldwide Health Organization
ZN	Ziehl-Neelsen

LIST OF APPENDICES

PENGAKTIFAN SEL DENDRITIK MANUSIA OLEH LIPOSOM DARIPADA KESELURUHAN LIPID *Mycobacterium smegmatis*

ABSTRAK

Liposom adalah lipid bersaiz kecil, kuat, dan tersusun sendiri yang mempunyai potensi besar sebagai penghantar ubatan dan adjuvan yang cekap. Ia mempunyai struktur vesikular yang unik dan boleh diperoleh daripada bahan semula jadi dan sintetik. Liposom boleh menyerupai membran biologikal ubat, hal ini demikian dapat memanjangkan jangka hayat dan mengurangkan tahap ketoksikan semasa menghantar mereka ke organ yang dituju. Mekanisme adjuvan liposom berkait rapat dengan rangsangan terhadap tindak balas imun yang diingini setelah terdedah kepada antigen dan sel imun yang dituju. Kajian semasa ini bertujuan untuk menyiasat pengaktifan sel dendritik manusia oleh liposom daripada keseluruhan lipid *Mycobacterium smegmatis*. Di sinilah, liposom yang dihasilkan daripada keseluruhan lipid *M. smegmatis* dan gambaran ciri di bawah dibawah mikroskop elektron pengimbasan pelepasan medan (FESEM) yang menunjukkan saiz liposom adalah di antara 20 nm-135 nm dengan pembentukan struktur sfera. Sampel keseluruhan darah manusia dikumpul dan diasingkan untuk mendapatkan sel mononuklear darah peripheral (PBMCs) daripada tiga kumpulan berbeza: individu negatif-TST, individu positif-TST, dan pesakit TB pulmonari aktif. Pengaktifan imun sel dendritik oleh liposom *M. smegmatis* dianalisis berdasarkan tahap ekspresi penanda permukaan sel dendritik (HLA-DR, CD11c, CD123, dan CD86) dalam sitometri aliran dan rembesan sitokin (IL-4, IL-12p70 dan IFN-γ) melalui ujian ELISA. Keupayaan liposom dalam menambah baik penyampaian antigen semasa kadar jangkitan TB yang aktif telah dibuktikan melalui peningkatan tahap HLA-DR dan CD86 bersama

dengan rembesan sitokin IL-12p70, IFN-γ, dan IL-4 yang tinggi. Pengesahan yang selanjutnya adalah melalui FESEM dan mikroskop konfokal yang memperlihatkan pengambilan liposom *M. smegmatis* oleh sel dendritik. Pendedahan liposom kepada sel dendritik dalam semua kumpulan kajian di bawah pengimejan FESEM menunjukkan pembentukan bulatan besar pada permukaan sel dendritik di mana ia menggambarkan kewujudan liposom pada permukaan sel dendritik berbanding dengan kawalan negatif dan positif. Penonjolan dendrit dengan pembentukan bentuk sel yang pelbagai dengan kehadiran perangsang membantu pengaktifan sel dendritik. Begitu juga, isyarat pendarfluor yang diperhatikan di bawah mikroskop konfokal menyokong internalisasi liposom oleh sel dendritik. Secara keseluruhannya, kajian ini mencadangkan liposom mempunyai potensi yang besar sebagai vaksin dan adjuvan yang berkesan untuk imunoterapi.

ACTIVATION OF HUMAN DENDRITIC CELLS BY LIPOSOMES DERIVED FROM TOTAL LIPID OF *Mycobacterium smegmatis*

ABSTRACT

Liposomes are small-sized, potent, and self-assembled lipids that hold great potential as efficient drug delivery vehicles and adjuvants. They possess a unique vesicular structure and can be derived from natural and synthetic substances. Liposomes can mimic the biological membrane of drugs, thus extending the half-life and minimizing the toxicity levels while delivering them to the target organs. The adjuvant mechanism of liposomes has been intimately associated with the stimulation of desired immune responses upon the exposure of antigen and immune cell targeting. This current study mainly targets to investigate the activation of human DCs by liposomes derived from the total lipid of *Mycobacterium smegmatis.* Herein, the liposomes were produced from *M. smegmatis* total lipid and characterized under field emission scanning electron microscopy (FESEM), demonstrating a size ranging from 20 nm-135 nm with spherical structures. The human whole blood sample was collected and isolated to obtain the peripheral blood mononuclear cells (PBMCs) from three distinctive groups: TST-negative individuals, TST-positive individuals, and active pulmonary TB patients. The immune activation of DCs by *M. smegmatis* liposomes was analysed by the expression level of DCs surface markers (HLA-DR, CD11c, CD123, CD86) in flow cytometry and the secretion of cytokines (IFN-γ, IL-12p70, and IL-4) and via ELISA assay. The capability of liposomes to improve the antigen presentation during the active state of TB infection was approved through the increased level of HLA-DR and CD86 alongside the high concentration levels of IL-12p70, IFN-γ, and IL-4 cytokines.

Further confirmation study via FESEM and confocal microscopy recognized the uptake of *M. smegmatis* liposomes by DCs. The exposure of liposomes to DCs in all study groups under FESEM imaging showed the large circular formation on the surface of DCs which pointed out the presence of liposomes on the surface of DCs compared to the negative and positive controls. The protrusion of dendrites with different cell-shaped development with the presence of different stimulators assisted in the activation of DCs. Similarly, the fluorescence signals observed under the confocal microscope supported the internalization of liposomes by DCs. Overall, this study suggests that liposomes have significant potential as effective vaccines and adjuvants for immunotherapy.

CHAPTER 1

INTRODUCTION

1.1 Rationale of the study

Tuberculosis (TB) remained in the top 10 worldwide diseases with a clear mortality impact and even surpassing HIV/AIDS has urged global public health priority (Schrager et al., 2020). Nearly a quarter of the worldwide population has been highly infected with TB infection which is associated with poverty as well as low and middle-income countries (Franco & Peri, 2021). Although Bacille Calmette-Guérin (BCG) is the only applicable and licensed vaccine for TB, it encountered several limitations such as genetic variability among strains that lead to differences in immunogenicity (Sable et al., 2019). In addition, it provides poorer protection among higher age groups due to their highly matured immune system that stimulates weaker responses to BCG in comparison to the sustained and increasing immune reaction in children (Bendre et al., 2021). These issues mainly emphasize the significance of immune cells such as dendritic cells in triggering the desired immune responses against TB infection.

Dendritic cells (DCs), the key regulatory cell of the immune system is well-known as the most efficient professional antigen-presenting cells (APCs) that initiate humoral and cell-mediated immune responses (Ness et al., 2021). They are mainly originated from bone marrow hematopoietic precursor cells and are characterized by their distinctive ‘tree-like’ or dendritic-shaped appearances (Castell-Rodríguez et al., 2017). Upon encountering infected pathogens, DCs functionalized in capturing, processing, and presenting the antigen to major histocompatibility complexes (MHC) class I and II molecules alongside the secretion of cytokine (Gil-Torregrosa et al., 2004; Patente et al., 2018). These

resulted in the activation of naïve T cells that further stimulate the appropriate adaptive immune responses by enhancing host defense mechanisms or facilitating the evasion of pathogens (Kim & Shin, 2022). The initial discovery of DCs by Ralph Steinman in the early 1980s concluded with the mechanical action of the immune system as a coherent unit (Mellman, 2013). Indeed, DCs are also responsible for maintaining 'self' tolerance by the induction of regulatory T cells (Tregs) and deletion of T cells. Therefore, it plays a crucial role in the pathogenesis of cancer and autoimmune diseases, such as type 1 diabetes mellitus (T1D), multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (Quintana, 2017). However, the interaction between DCs and *Mycobacterium tuberculosis* (Mtb) is not fully understood and is contradictory despite its high potential to improve cellular immune response against Mtb (Choi et al., 2018; de Martino et al., 2019; Zhou et al., 2023).

Immunity mediated by $CD4^+$ and $CD8^+$ T cells is highly essential for the defense against TB infection. Although most of the infected individuals who develop latent infection remain healthy throughout their whole lives, nearly 5-10% of the cases are highly potential to progress into the active state of TB. Earlier research demonstrated strong T-cell responses to the protective Mtb heparin-binding hemagglutinin (HBHA) only within latent TB individuals (LTBI) in comparison to active TB patients, enhancing the dissemination of Mtb from the site of infection (Masungi et al., 2002; Temmerman et al., 2004). The FcγRIII (CD16) receptor, which is involved in Ab-dependent cellular toxicity, is more active in LTBI than active TB and this eventually promotes an improved phagolysosome maturation, increased inflammasome activation, with reduced mycobacterial burden (Lu et al., 2016). In addition, active TB is highly dependent on pro-

inflammatory immune responses (Flores-Batista et al., 2007). The suppression of T-cells particularly in active TB implies the variation of functional DCs between healthy individuals, LTBI, and active pulmonary TB patients, emphasizing the regulatory mechanism of immune evasion by Mtb. This highlights the demand to grasp a full understanding of the host immune response against Mtb to differentiate between normal and pathogenic processes. Henceforth, expressing the need for an adjuvant or carrier that potentially targets and improves the activation of DCs to trigger desired immune responses against Mtb.

Liposomes are small sphere artificial vesicles with an aqueous solution core, surrounded by a membranous lipid bilayer of phospholipids made up of natural or synthetic derivatives (Akbarzadeh et al., 2013). The nanoscale size of liposomes is ranging between 25 to 2500 nm. High versatility with minimum effects on the immunogenic and toxicity are the main advantages (Mallick & Choi, 2014). Thus, recognizing liposomes as the most actively used nanocarriers for targeted drug delivery at present (Alavi et al., 2017; Gregoriadis, 2016). Previous *in vivo* research had elucidated the potent uptake and activation of human DCs upon interactions with designated liposomes derived from *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), *Archaea,* and *Escherichia coli* (*E. coli*) (Sprott et al., 2004). These were achieved by the immunostimulatory properties of liposomes (Amidi et al., 2011). Nevertheless, relevant studies on the liposomal-based derived from the total lipid of *Mycobacterium smegmatis* (*M. smegmatis*) have not yet been scrutinized. In another designated novel smegmosomes study, strong induction of both innate and adaptive immune responses by bone-marrow-derived DCs (BMDCs) was simultaneously demonstrated (Faisal et al., 2011). However, the utilization uptake mechanism of DCs was not justified. With that, the interest to investigate further the activation of

DCs upon *in vitro* uptake by *M. smegmatis* liposomes in this context is highly considered.

As mentioned above, liposomes are usually produced from the derivatives of natural (soybean or bean) or synthetic sources (Nkanga et al., 2019). However, due to the pricey and limited sources of liposomes lipid bilayers, this study sought another alternative. In a previous recent successful development of new potential tuberculosis (TB) vaccine candidate, liposomes-Msmeg, ranging between 20 to 80 nm, had manifested a conveying outcome upon Mtb antigens (García Mde et al., 2014). Initially, this designated liposome was developed using the bacterial lipid synthesis of *M. smegmatis* due to its high resemblance of genomic manipulation, chromosome arrangement, and similarities in the cell wall composition to Mtb (Joseph Antony Sundarsingh T et al., 2020). These genetic correlations enable further investigation on TB infection to be performed using the non-pathogenic *M. smegmatis* model organism which provides a safer, accessible, and rapid duration of culture, as a substitute for the highly pathogenic Mtb (J. A. S. T et al., 2020; Yamada et al., 2018). The preliminary results had emphasized positively the activation and maturation markers of murine DCs by liposomes Msmeg fusion, with induced *in vitro* immune responses. Thus, this present study has attempted to examine the phenotype and functional properties of DCs in TST-negative individuals, TST-positive individuals, and active pulmonary TB patients to induce immune activation upon exposure to liposomes derived from total lipid of *M. smegmatis*. This further suggests its capability as a potential adjuvant, carrier, or vaccine against TB infection.

1.2 Research objectives

1.2.1 General objective

To investigate the activation of human dendritic cells by liposomes derived from total lipid of *Mycobacterium smegmatis*

1.2.2 Specific objectives

1) To produce liposomes from the total lipid of *M. smegmatis*

2) To determine the dendritic cells subsets in human peripheral blood mononuclear cells of TST-negative individuals, TST-positive individuals, and active pulmonary TB patients

3) To measure the expression marker of HLA-DR, CD11c, and CD86 in activated dendritic cells from human peripheral blood mononuclear cells upon exposure to liposomes from *M. smegmatis* in TST-negative individuals, TST-positive individuals, and active pulmonary TB patients

4) To measure the secretion levels of IFN-γ, IL-12p70, and IL-4 in dendritic cells from human peripheral blood mononuclear cells upon exposure to liposomes from *M. smegmatis* in TST-negative individuals, TST-positive individuals, and active pulmonary TB patients

5) To examine the cellular uptake of liposomes from *M. smegmatis* by dendritic cells from human peripheral blood mononuclear cells in TST-negative individuals, TST-positive individuals, and active pulmonary TB patients

1.3 Research hypothesis

This study hypothesized that the production of liposomes from *M. smegmatis* will be taken up by DCs and alter their immune activation in PBMCs of TST-negative individuals, TST-positive individuals, and active pulmonary TB patients.

1.4 Flow chart of the study

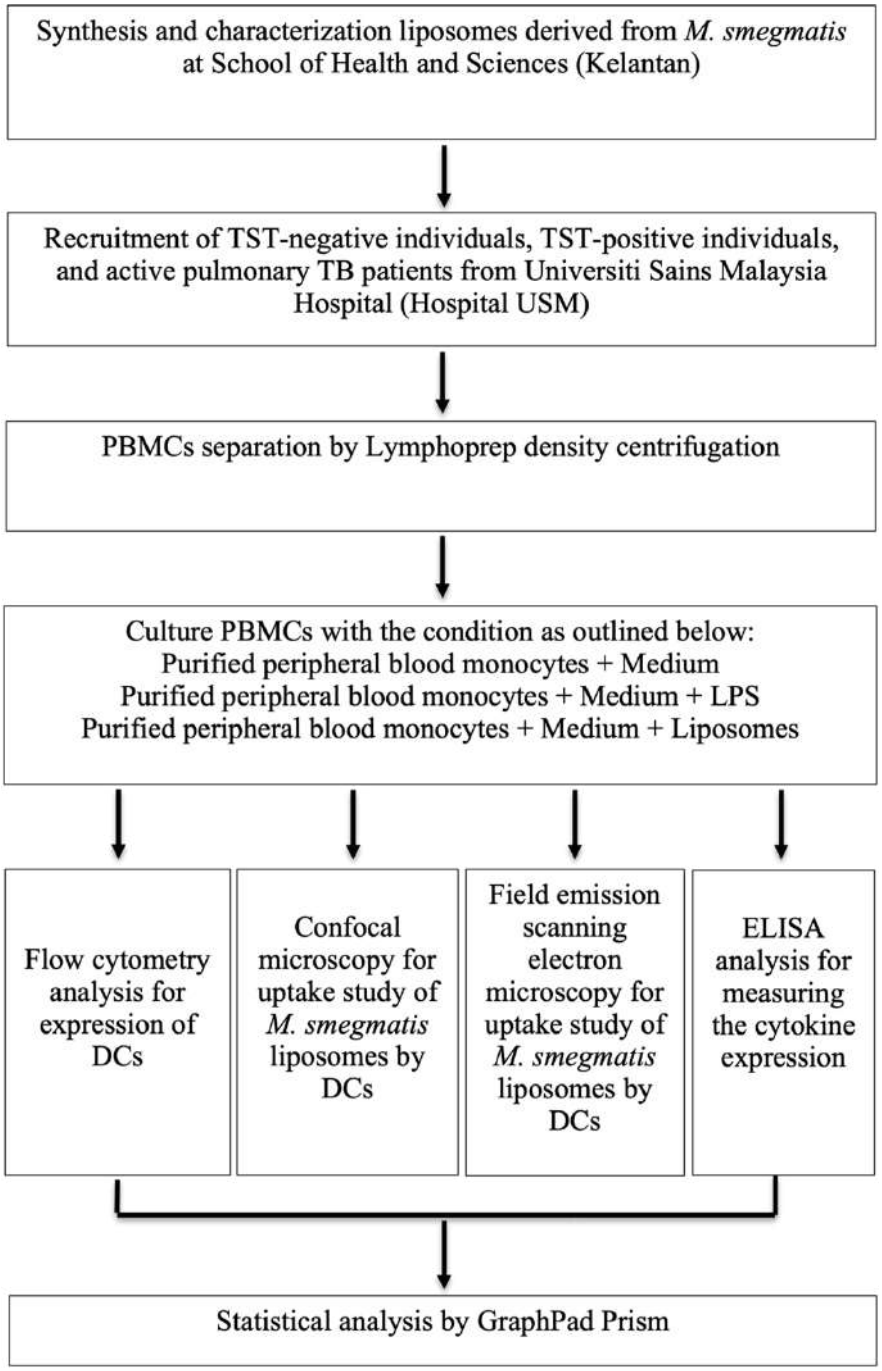

Figure 1.1 Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is one of the most infectious progressive mycobacterial infections, affecting the lungs (pulmonary TB) (Daniel et al., 1994). The cycle of TB infection exclusively begins with aerosol inhalation of Mtb pathogen from coughing and sneezing droplets (Figure 2.1) (Churchyard et al., 2017; Glickman & Jacobs, 2001). The risk factors associated with TB mainly include close-contact situations, individuals with a weakened immune system (e.g., human immunodeficiency virus (HIV)), malnutrition, tobacco smoke, substance abuse, use of immunosuppressive drugs, poverty, young children and elderly populations, and healthcare workers, all of which contribute to poor treatment results (Narasimhan et al., 2013). Therefore, pressing the need to study and evolve new prevention and therapeutic strategies for TB. There are two distinct categories of TB, namely latent TB infection (LTBI) and active pulmonary TB (Brett et al., 2020). Individuals with LTBI are not contagious due to the absence of visible symptoms, unlike active, contagious TB (Gideon & Flynn, 2011). Pulmonary TB commonly manifests prolonged bad cough (exceeding two weeks), chest pain, haemoptysis, weight loss, fatigue, sweats, and fever (Cudahy & Shenoi, 2016). Sputum-smear status is highly associated with pulmonary TB (Ibrahim et al., 2022). Bacillary load at the site of diagnosis (i.e., sputum or lung cavities), and the exposure to a high dose of viable Mtb favours more efficient chances of transmission, which can be rapidly suppressed with proper effective treatment (Kolloli et al., 2021; Osei-Wusu et al., 2021).

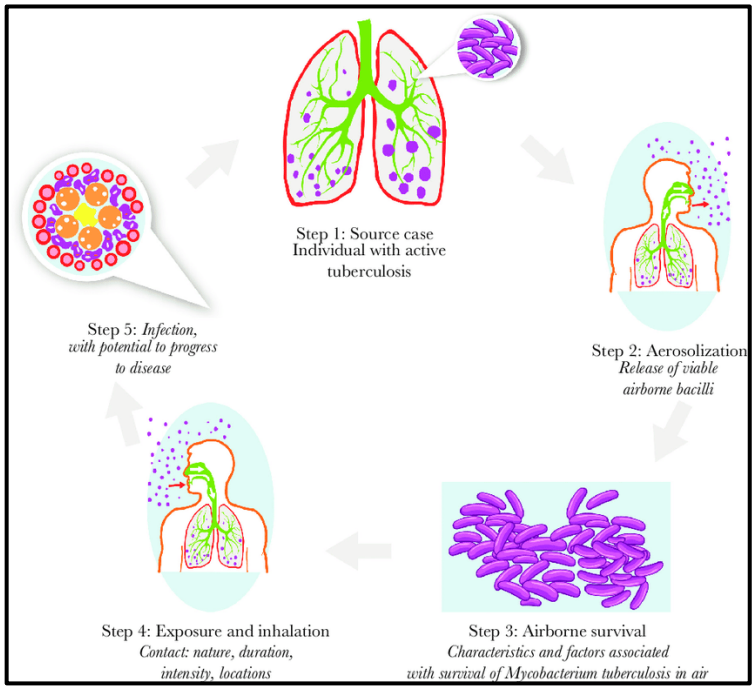

Figure 2.1 The transmission of TB infection (Adapted from: Churchyard et al., 2017)

2.1.1 Tuberculosis cases worldwide

Tuberculosis was the leading illness with a high mortality rate especially during the initial breakdown, in the late 1800s. Administrative, environmental, and preventive measures such as implementing a written TB infection-control plan, proper disinfection of equipment, and good hygiene practice have reduced the number of TB cases and deaths, significantly (da Costa et al., 2009). Overall, the highest cases of TB infection mainly occurred within the WHO regions of South-East Asia (43%), Africa (25%), Western Pacific (18%), Eastern Mediterranean (8.3%), America (3.0%), and Europe (2.3%). According to the World Health Organization (WHO) report for the year 2021, an estimation of 10.6 million people has been diagnosed with TB infection, with 1.2 million deaths among HIV-negative people (Chakaya et al., 2021). Out of the 10 million reported cases, 6 million were mainly comprised men, followed by 3.4 million women and 1.2 million children (Figure 2.2). The new cases of TB globally fell from 7.1 million (2019) to 5.8 million

(2020), which was a decline of nearly 20% compared to the average number of cases reported within 2016-2019 (WHO, 2021). The major coronavirus disease 2019 (COVID-19) outbreaks and health care service disruption have led to movement control order (MCO) implementation, reducing the transmission of TB infection among travelers from the high-incidence regions (Pai et al., 2022; Winglee et al., 2022). The countries contributing to the global drop between 2019 and 2020 were India (41%), Indonesia (14%), the Philippines (12%), and China (8%). In contrast, the TB mortality rate in the year 2020 had increased for the first time in more than a decade with an approximation of 1.5 million cases (WHO, 2021). This issue has also been highly related to the COVID-19 pandemic. High expenses caused by TB disease on both human and financial resources give an impact on economic growth and are conductive to improve public health (Miggiano et al., 2020).

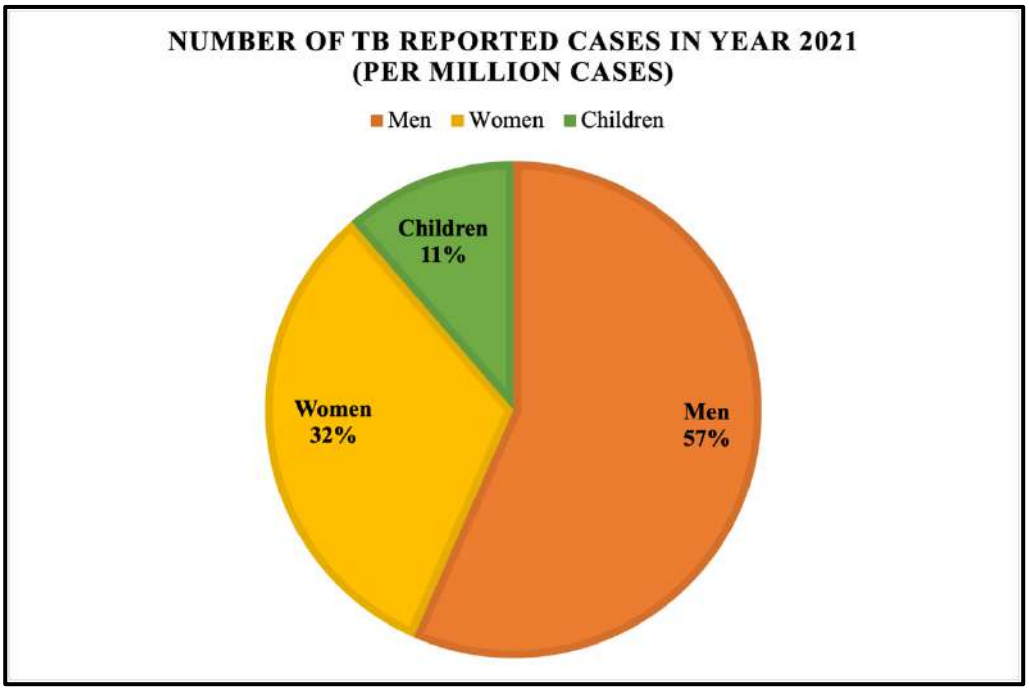

Figure 2.2 The statistical report of TB cases by the World Health Organization (WHO) for the year 2021

2.1.2 Tuberculosis cases in Malaysia

The rate of TB transmission in Malaysia remains active with an intermediate TB burden despite the improved treatment regimens and case detection applied (Awang et al., 2022). Collection report from World Bank in the year 2020 showed that the incidence rate of TB in Malaysia was 92 (per 100, 000 population) with four cases (per 100, 0000 population) of TB mortality rate (Figure 2.3) (Avoi & Liaw, 2021). Currently, approximately 53% of TB cases in Malaysia mostly occurred in the adult group with high prevalence in other groups including children, adolescents, and the elderly (Awang et al., 2022). For instance, the proportion of pediatric TB cases in Kelantan state showed an increasing trend from 1% to 8.4% from the year 2000 to 2019 (Awang et al., 2019). The incomplete development of immunity, especially in children aged <5 years old highly contributes to this event (Azit et al., 2019). Selangor and Sabah led with the highest number of TB incidences in the year 2018; 5 071 cases and 5008 cases, respectively. These were mainly due to the large density of the Selangor populations in the low socioeconomic rungs and the presence of illegal immigrants who refuse to seek treatment with the limited access to TB care in Sabah (Rundi et al., 2011). The Selangor state, the most populated state comprises nine districts with four bigger districts (i.e., Hulu Langat, Gombak, Petaling, and Klang) leading to almost 18% of the overall cases in the country (Makeswaran et al., 2022). On the other hand, Sabah state which only accounts for 10% of the total population in Malaysia had sustained a high notification rate, approximately 20-30% of all TB cases in Malaysia (Goroh et al., 2020). Other states contributing to newly reported TB cases were Sarawak, 3122; Johor, 2150; and Kuala Lumpur, 2017 cases. The mortality rate of TB in Malaysia increased significantly from 9.0 per 100 000 people (2014) to 11.4 per 100 000 people (2018) over the last four years (Avoi &

Liaw, 2021). Most of the active TB deaths were highly correlated to disseminated TB, whereas non-TB-related deaths occurred due to the existing comorbidities.

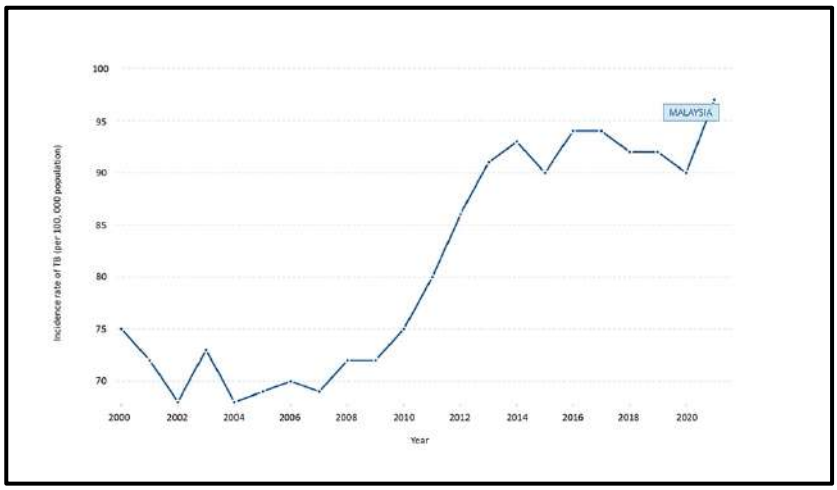

Figure 2.3 The incidence rate of TB (per 100,000 people) in Malaysia 2000-2021 (Adapted from: World Health Organization, Global Tuberculosis Report)

2.1.3 Pathogenesis of tuberculosis

Transmission of Mtb occurs through the inhalation of aerosolized particles ranging from 0.65 μm to 7.0 μm (Sia & Rengarajan, 2019). Smaller Mtb droplets travel into the nasopharyngeal region to be deposited in distal airways, meanwhile, larger aerosol particles remain in the upper airways (Bussi & Gutierrez, 2019). The mucous lining of the human nasal and respiratory tract surface act as the first line of defense to inhibit the entry of Mtb into pulmonary alveoli (Mohidem et al., 2021).

Once reaching the lower respiratory tract, immature DCs mainly identify, phagocytes, and internalize Mtb bacilli into phagosomes (Figure 2.4). The engulfed Mtb is breakdown into simpler peptide fragments and is presented on the surface of DC, specifically through MHC II molecules. This leads to the maturation and activation of DCs, allowing subsequent migration to present the Mtb-derived peptide to the nearby lymph nodes where the naïve T cells are located. The differentiation of naïve T cells effectively primes antigen specific CD4 and CD8 T cells (Ahmad,

2011; Wani, 2013). Alongside the presence of IL-12p70 cytokine, it promotes the differentiation of $CD4^+$ T cells into Th1 cells that resulted in the secretion of their signature IFN-γ cytokine. This pathway is commonly known as the classical macrophage activation (M1) which stimulates the phagocytosis of the macrophage and intracellular mycobacterial killing. Simultaneously, the canonical type 2 cytokines, namely interleukin 4 (IL-4) together with IL-13, could activate macrophages through alternative macrophage activation (M2), enhancing protective innate memory and killing capacity against mycobacterial. Macrophages showed a crucial role in engulfing and destroying the Mtb, yet the mechanism for survival of mycobacterium within the macrophages is evolving progressively. This leads to the induction of immune response including the recruitment of other immune cells including T cells.

The infected macrophages are surrounded by phagocytic cells including uninfected alveolar macrophages, lymphocytes, neutrophils, and monocytes (Flynn et al., 2011). This ultimately leads to the formation of nodular granulomatous, the complex structures that wall off the infection from spreading and represented as the hallmark of TB disease (Orme & Basaraba, 2014; Silva Miranda et al., 2012). In this state, the granuloma secures Mtb bacilli by putting it into an inactive phase or LTBI, thus no symptoms are presented (Ehlers & Schaible, 2012). A caseous granuloma that illustrates a ‘cheese-like’ appearance, has been commonly known as the classic type of granuloma in TB (Flynn et al., 2011). The advancement of LTBI towards active TB is highly associated with the multiple depositions of the caseum that necrotizes the center of granuloma (Ehlers & Schaible, 2012). Immune weakening conditions such as stress, malnutrition, and immunosuppressive medications could

lead to the replication of Mtb within the granuloma and destruction of lung tissue, reactivating and releasing Mtb into the airways expedites TB infection.

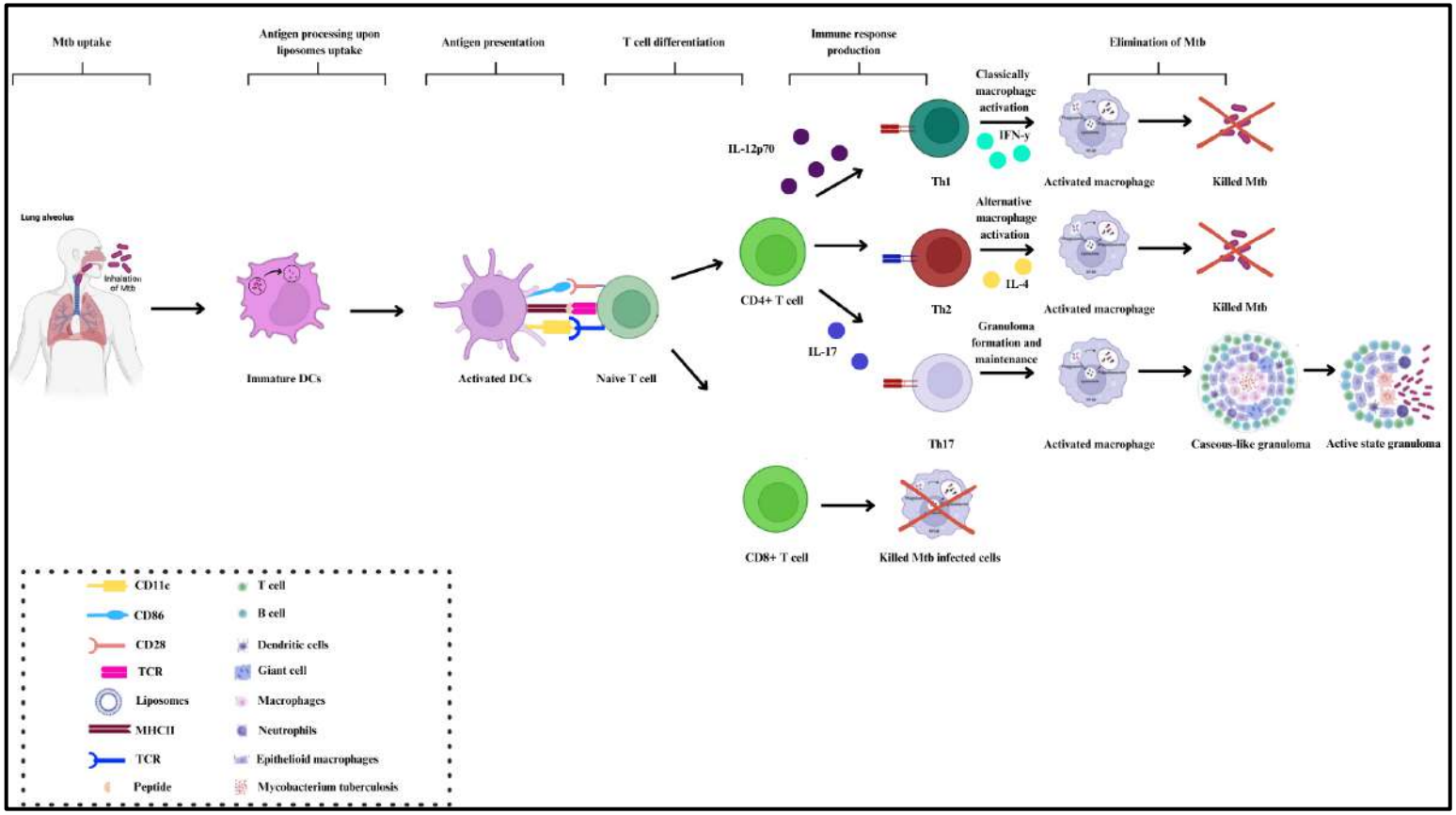

Figure 2.4 Pathogenesis of tuberculosis

The inhalation of Mtb pathogen leads to the phagocytosis process of macrophages and DCs. The exposure of Mtb allows the immature DCs undergoes maturation and send signals to differentiate naïve T cells into $CD4^+$ and $CD8^+$ T cells. The $CD4^+$ T cells prime Th1, Th2, and Th17 responses, whereas $CD8^+$ T cell leads to the killing of Mtb infected cells. The stimulation of the IL-17 cytokine by Th17 cells assists in granuloma formation. The death of infected cells causes disintegration of granuloma, releasing Mtb pathogen into lung airways and environment.

2.1.4 Diagnosis and treatment of tuberculosis

Diagnosing TB is a complex process that begins with a history tracing and the likelihood of being exposed to any active individual (Norbis et al., 2013). A series of screening tests must be performed to confirm the status of TB and the treatment needed. Mantoux tuberculin skin test (TST) has been widely used as a method to determine Mtb infection within individuals (Figure 2.5) (Rose et al., 1995). The result is analysed based on the size of induration that appears within 48 and 72 hours, upon the administration of tuberculin purified protein derivative (PPD) into the inner surface of the forearm (Rose et al., 1995). Since the symptoms of TB may vary depending on the site of Mtb growth, a blood test needs to be executed as it provides conclusive results in ruling out LTBI and active TB, based on the immune system reaction against Mtb. Radiology evidence such as chest X-rays, could provide early detection of lung abnormalities associated with TB, especially among children (Heuvelings et al., 2019). Mycobacterial culture has been regarded as the 'gold standard' in drug susceptibility tests for TB as it provides a definite accurate diagnosis (Holani et al., 2014; Shingadia & Novelli, 2003).

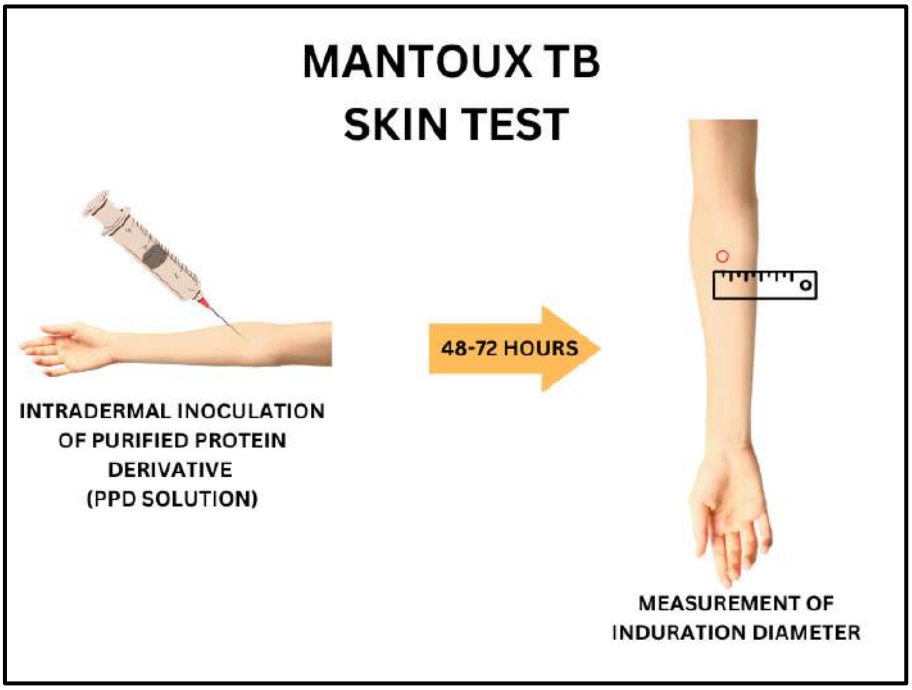

Figure 2.5 Mantoux test for TB screening

Early detection and appropriate antibiotics are crucial for TB infection. The current treatment for TB clinically relies on a drug that exhibits early bactericidal activity (EBA) such as isoniazid (INH) (Peloquin & Davies, 2021). The usage of combination drugs is highly preferable in treating TB infection rather than the prolonged treatment using a single drug, due to the emergence of resistant mutants (Kaneko et al., 2011). INH and rifampicin are two excellent duos that work best at the former and latter stages, respectively (Maiolini et al., 2020). However, the rifampicin drug has a slower drug interaction that called for long-term intake which further required its substitution with the rifapentine drug that could exhibits a synergistic mechanism and results in improved and excellent antibacterial properties (Alfarisi et al., 2017). Besides, the prescription of suitable TB regimens depends on the stages of TB, which occur either during exposure, latent, or active disease as well as the specificity action and effect of the antituberculosis drugs (Sotgiu et al., 2015). The usage of a regimen for multidrug-resistant TB (MDR-TB) is highly required for complex cases such as drug intolerance, where the infected individuals remain

sputum smear-positive upon completion of treatment (Rabahi et al., 2017). A previous study using line probe assay (LPA) supported the administration of a standardised second-line anti-TB drug regimen as an alternative to the ineffective MCR-TB cases (Raizada et al., 2014). The usage of a second-line anti-TB drug regimen which comprised bedaquiline (Bdq), linezolid (Lzd), moxifloxacin (Mfx), levofloxacin (Lfx), clofazimine (Cfz), cycloserine (Cs), para-aminosalicylic acid (PAS), propylthiouracil, and amikacin (Am) exhibited good potential to alter the structural composition of the intestinal microbiota in rifampicin-resistant TB patients (RR-TB) (Wu et al., 2023). These highlighted the importance of providing the suitable anti-TB treatment in each diagnosis.

2.2 Immune response

Immunity can be defined as a condition where the host defense system is activated to protect the body against the pathogenic microbes, viruses, non-pathogenic agents, and foreign substances that could lead to infection (Sompayrac, 2019). This further leads to the manipulation of normal cellular function that trigger tissue inflammation, evasion of immune responses, and interference in the host defense system, highlighting the critical function of immunity (Thakur & Nanda, 2020). Two types of human immune systems are the innate (non-specific) immune system and the adaptive (specific) immune system (Medina, 2016). Innate immunity steps up as an early and rapid defence upon encountering immediate intruding pathogens with no immunologic memory (Marshall et al., 2018). Meanwhile, adaptive immunity is an antigen-dependent that stimulates highly efficient and targeted responses of antibody production and cell-mediated responses of specified

pathogens (Han et al., 2020). Both are complementary to one another in defending the body against foreign antigens.

2.2.1 Innate immune response

The first line of defense found in the body is the innate immune system, also known as a non-specific defense mechanism (Figure 2.6) (Nicholson, 2016). It is essentially composed of physical barriers (skin and mucous membrane), chemical barriers through the action of antimicrobial peptides and proteins (i.e., complement, C-reactive proteins), as well as cellular components that release cytokines and inflammatory mediators (e.g., macrophages, DCs, natural killer (NK) cell) (Carrillo et al., 2017; Sharpe & Mount, 2015). The pivotal role of this rapid non-specific response is to provoke immediate protection throughout the body to halt the spread of foreign pathogens (Sompayrac, 2019). It is achievable by the action of cytokines and chemokines, which resulted in the rapid recruitment of immune cells to the sites of infection and inflammations.

The presence of microbial agents is primarily detected by specialized pattern recognition receptors (PRRs), a broad family of proteins, which are expressed by the innate immune effector cells (e.g., DCs, macrophages, mast cells) (Clark & Kupper, 2005). There are mainly four classes of proteins categorized by PRRs, with toll-like receptors (TLRs) playing a critical role in early innate immunity by the detection of invading microorganisms and danger signals. Toll-like-receptor is the most studied family of PRRs (Mogensen, 2009). They act through the recognition of conserved microbial structure, via pathogen-associated molecular patterns (PAMPs) (Jang et al., 2015). Specific intracellular signalling pathways are further activated, thus triggering proinflammatory and antimicrobial responses (Gasteiger et al., 2017). On the other hand, damage-associated molecular patterns (DAMPs) are characterized as

endogenous molecules released upon cellular injury (Land, 2015). The interaction between DAMPs and PRRs eventually promotes an innate immunity activation during sterile inflammation (Černý & Stříž, 2019; Roh & Sohn, 2018).

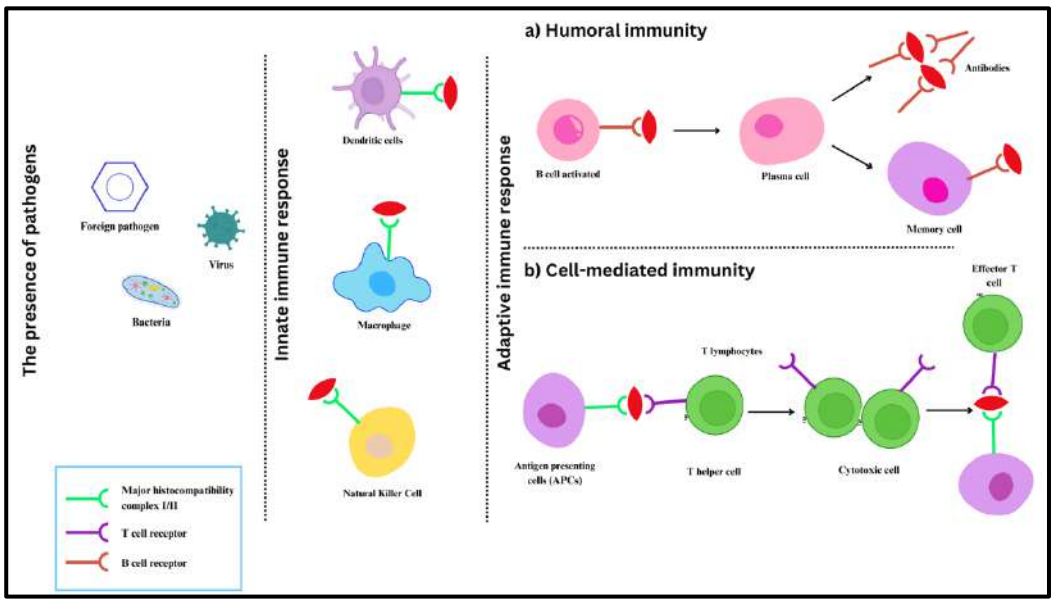

Figure 2.6 The subsystems of immune responses: Innate and adaptive immunity

2.2.2 Adaptive immune response

Adaptive immune systems are mainly referred to as antigen-specific and antigen-dependent immune responses (Figure 2.6) (Marshall et al., 2018). It is considered the second line of defense against nonself-pathogens and is more sophisticated than innate immunity, due to the presence of memory cells (Snyder, 2017). Therefore, it comprises the memory capacity which facilitates an efficient elimination response against the recurrent pathogen-infected cells within an instant (McManus & Mitchell, 2014). Adaptive immunity mainly provides longer protection. There are fewer cells involved in adaptive immunity: B cells, which differentiate into plasma cells and produce antibodies, as well as antigen-specific T cells that proliferate upon direct interaction with APCs (Bonilla & Oettgen, 2010). Both lymphocytes are derived from the multipotent hematopoietic stem cells of bone

marrow. Two mechanisms of adaptive responses include humoral immune response and cell-mediated immune response.

Humoral immunity is an antibody-mediated response against extracellular pathogen, viruses and bacteria which circulate in the lymph or blood. It is primarily driven by B cells that activated into plasma cells, resulting in the production of antibodies against specific pathogens (Shah & Ershler, 2007). There are three defensive mechanisms performed by antibodies to destroy the invading pathogens (Forthal, 2014). Neutralization is the first elimination method, which involves the inhibition of microbial toxins and the inactivation of viruses by antibody binding onto pathogens. The subsequent process, namely opsonization, is referred to as antibody binding by coating the invading pathogens for destruction through phagocytosis, mostly to aid phagocytic cells for ingestion. The third approach in antibody defense is the activation of the complement cascade, which results in phagocytic cell recruitment (Sawa et al., 2019). Humoral immunity can only protect against most bacterial and viral toxins in the extracellular spaces (Twigg, 2005). Thus, another layer of defense is highly required to act on intracellular microbes (e.g., parasites, tumour cells, bacteria).

Cell-mediated immunity is an immune response involving both $CD4^+$ and $CD8^+$ T cells, leading to the activation of APCs such as macrophages and DCs, stimulation of antigen-specific cytotoxic T lymphocytes, and secretion of diverse cytokines in response to antigens (Figure 2.4) (Bhagavan & Ha, 2011). $CD4^+$ T cells or T-helper (Th) lymphocytes are known as the effector cells for cell-mediated immunity. They primarily recognized protein-based antigens presented by MHC II molecules on the surface of APCs, coordinating the stimulation of different subtypes

of $CD4^+$ T cells: so-called Th1, Th2, and Th17 lymphocytes (Wichmann & Angele, 2010). The polarization of Th1 is mainly induced by IL-12 cytokine, whereas IL-4 is accountable for Th2 polarization. The Th1 response further secretes IFN-γ cytokine that will act correspondingly on different types of immune cells, with each enhancing their own effector functions (Marshall et al., 2018). Meanwhile, the Th2 response which secretes IL-4, IL-10, IL-5, and IL-13 cytokines is more likely to be crucial for the defense against extracellular parasites and pathogens, orchestrating allergic reactions, in conjunction with the development of humoral immune responses (Xu et al., 2019).

The $CD8^+$ T cells which are also called cytotoxic T lymphocytes (CTLs) upon activation have been an important effector for the elimination of viral infected cells and cancerous pathogens (Xu et al., 2023). Upon encountering the antigen processed and presented by activated DCs through MHC I peptide complex, the CTLs primarily kill the targeted cells by apoptosis (Actor, 2019). In addition, CTLs can produce type I cytokines such as IFN-γ, TNF-α, and lymphotoxin-α, supporting its role as one of the critical immune cells for the control of viral and other intracellular infections (Leichner & Kambayashi, 2014).

2.2.3 Immune response to tuberculosis

The encounter between Mtb and the host is a complex process that will determine whether the TB infection will remain locally limited within the engulfing cells of the innate immune system (LTBI), continue to spread (active TB), or complete clearance of Mtb pathogen (de Martino et al., 2019). Interaction between T cells, macrophages, and granuloma formation is the pillar component of the protective response against Mtb (Figure 2.7) (Tufariello et al., 2003).

The immune response is initiated upon the entrance of Mtb bacilli into the alveolar space (Basu et al., 2012). Phagocytic cells such as macrophages and immature DCs are highly represented in the infected area of TB (Mihret, 2012). Pathogen-associated molecular patterns (PAMPs), which are antigenic compounds expressed by Mtb are mainly recognized by a large of pattern-recognition receptors (PRRs) including toll-like receptor (TLRs) on macrophages and DCs. These receptors facilitate the pathogen-specific ligand, allowing immature DCs to uptake the Mtb and undergo antigen processing by breaking down the antigen into peptide fragments. The DCs then mature, migrate from the lungs to the regional lymph nodes (LNs) and present the Mtb antigen (peptide) onto its surface to the T cells, via both MHC class I and II pathways (Basu et al., 2012). T cells are activated and travel back to the lungs through the bloodstream. Within the lungs, T cells are later associated with the granuloma formation and assisted the macrophages for intracellular killing of Mtb, through the secretion of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Marino & Kirschner, 2016). Meanwhile, proinflammatory cytokines such as TNF-α, IL-1β, IL-12, and IL-6 are secreted through mycobacterial recognition of PRRs, sending signals to other immune cells to maintain and control the Mtb infection (Lin et al., 2007; Mortaz et al., 2012). The TNF-α further induces the downstream signalling nuclear transcription factor (NF)κ-B pathway (Fallahi-Sichani et al., 2012). At this stage, the phagolysosome engulfs the Mtb and fuses with lysosomes. This fusion creates a mature phagolysosome with nuclear factor kappa B (NFκ-B) which regulates the release of the lysosomal enzyme such as reactive oxygen species (ROS) and hydrolytic enzymes to kill and digest the Mtb (Ramachandra et al., 2005). The inability of the host to eradicate Mtb will eventually preserve the infection as an

inactive state, where infected macrophages migrate into the tissue. The latter induces inflammatory mononuclear cells such as monocytes, lymphocytes, DCs, and neutrophils to travel to the site of infection and accumulate the granuloma formation (Pai et al., 2016; Portnoy et al., 2001).

However, Mtb has its escape mechanisms where it can block intracellular degradation and convert the hostile environment into a safer condition. Mtb and other slowly growing mycobacteria (such as *Mycobacterium bovis* and *Mycobacterium avium*) alter the setting of phagosome to survive within the macrophages (Ramachandra et al., 2005). A protein secreted from Mtb such as early secretory antigen 6/culture filtrate protein and ATP1/2 suppresses the acidification of phagosomes, which eventually increases the acidic environment to pH 6.2 and inhibits maturation of phagosome (Deretic & Fratti, 1999; Zhai et al., 2019). The pathogenic Mtb also averts the lysosomal pathway as a defensive tool against autophagy by exploiting Coronin 1 protein (Jayachandran et al., 2007; Saha et al., 2020; Seto et al., 2012). In addition, Mtb uses its excellent antioxidant system to overwhelm the ROS level of infected Mtb, supporting its survival and replication within the host (Mori & Pieters, 2018; Shastri et al., 2018). Other alternate adaptation pathways of Mtb to evade host immune response are by escaping into the cytoplasm and lipoprotein inhibition via TLR2 dependent manner (Hu & Spaink, 2022). This eventually causes the Mtb to exit the granuloma and further disseminate to form lesions, subsequently (Maphasa et al., 2020).

Granuloma is a complex and compressed structure, known as the major histopathological characteristic of TB (Figure 2.4). In this state, foamy and epithelial macrophages as well as multinucleated giant cells (MCG) which are fused from

monocytes, will align together to form a core within the infected macrophages (Lay et al., 2007; Silva Miranda et al., 2012; Volkman et al., 2004). Once it has formed, they are surrounded by a rim of T cells and the recruitment of macrophages and highly differentiated cells such as multinucleated giant cells, epithelioid cells, and Foamy cells (Kim et al., 2010; Peyron et al., 2008). Replication of Mtb takes place within the matured granuloma. Indeed, the secretion of TNF-α and IFN-γ are crucial to maintain the balance and contain the dormant Mtb, together with the production of anti-inflammatory IL-10 (Redford et al., 2011). Within the tuberculous granuloma, the development of central caseation necrosis (cheese-like appearance) has been observed (Bhavanam et al., 2016; Kim et al., 2010; Ulrichs & Kaufmann, 2006). The caseous centre is comprised of dead macrophages, remnants of infected cells as well as accumulated debris (Russell et al., 2009). If the balance of granuloma load is intolerable such as HIV infection, malnutrition, and genetic factors Mtb bacilli will be reactivated and dispersed (Silva Miranda et al., 2012). They can either re-enter the blood or be released into the airways through the respiratory tract (Shaler et al., 2013). This resulted in TB manifestation and is classified as an active TB disease.

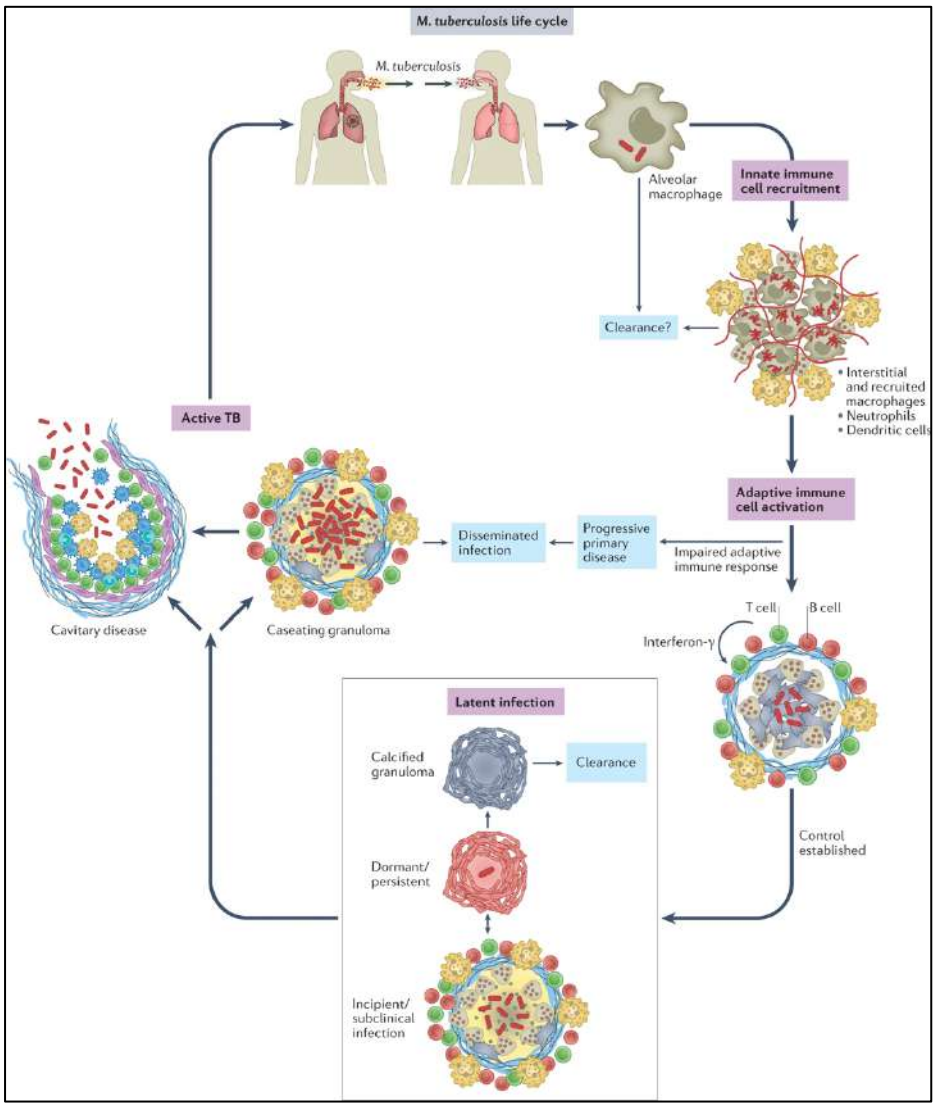

Figure 2.7 Interaction between innate and adaptive immunity in the presence of Mtb (Adapted from Chandra et al., 2022)

2.3 Dendritic cells

Dendritic cells are mainly characterized by a distinctive morphology of having long membranous dendrites (Figure 2.7) (Castell-Rodríguez et al., 2017). The origin of DCs can either be developed from common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) (Castell-Rodríguez et al., 2017). This eventually leads to the various lineages of DCs, namely myeloid (mDCs), plasmacytoid (pDCs), and monocytes-derived (mo-DCs) (Klechevsky et al., 2009). The common areas in which DCs are dominating may include the blood, lymphoid

and peripheral tissues. The mDCs are characterized by MHC class II and CD11c expressions, meanwhile, pDCs express MHC class II, BDCA2, and BDCA4 (Collin & Bigley, 2018; Merad et al., 2013). They are accountable for inducing an adaptive immune response and hence are centralized as the "sentinels" in immunity. Human circulating PBMCs are comprised of 1-2% of DCs, which is the most potent and professional APCs in comparison to other classical APCs such macrophages and B cells (Jongbloed et al., 2010). It stands out for its ability to stimulate, correlate, and initiate adaptive immune responses by activating both T and B cells (Patente et al., 2018). In addition, DCs are the only APCs of the immune system required for the activation of naïve T cells, both *in vitro* and *in vivo* (Makala & Nagasawa, 2002). This is mostly due to the highest competency to express MHC class II molecules, which further prime T cell responses (Sung, 2008). The secretion of IL-12 cytokines by DCs is critical to mediate Th1 adaptive immunity (Mendelson et al., 2006).

One of the critical roles of DCs is to interconnect the innate and adaptive immunity of the immune system (Ganguly et al., 2013). They are primarily equipped with long membrane dendrites that promote extensive communication with numerous surrounding cells (Swetman et al., 2002). The cells that can be reached out by DCs may include T cells, epithelial cells, and NK cells. Two mechanisms that highlight its 'sentinels immunity' include i) efficient presentation of antigens for the selection of appropriate T reactions, together with, ii) the detection of environmental signals by maturated DCs (Cechim & Chies, 2019). The differences in helper T cells produced by either Th1 or Th2 responses are mainly influenced by the types and maturation of DCs, stimulated by various factors.

Another interesting role of DCs is “cross-presentation”. This cross-priming process mainly involved the presentation of extracellular antigens with class I MHC molecules to $CD8^+$ cytotoxic T cells against foreign pathogens and tumors (Nierkens et al., 2013). There are two primary pathways involved in cross-presentation. First is the ‘canonical’ endosome-to-cytosol pathway which entails the transportation of exogenous antigens from endosomal vesicles into the cytosol, in which they are similarly processed and loaded on MHC I class molecules in the endoplasmic reticulum to endogenous antigens by proteosome (Hoeffel et al., 2007). The proteosome-independent cytosol-independent pathway is the second pathway where the DCs process and directly load the captured antigens onto MHC I class molecules in endosomal compartments (Di Pucchio et al., 2008). The efficient performance of cross-presentation is necessary to induce adaptive immunity against tumours and viruses that do not initiate a direct attack on DCs, instead, affecting those that cause harm to cells of peripheral tissue (Embgenbroich & Burgdorf, 2018; Sánchez-Paulete et al., 2017). This subsequently contributes to the generation of the cytotoxic immune response through protein antigens in tumour vaccines.

In addition, DCs also have the responsibility to modulate balances between immunity and tolerance (Patente et al., 2018). Immune tolerance is important for the prevention of self-attack which could result in autoimmune (Audiger et al., 2017). Tolerogenic DCs (tol-DCs) have crucially participated in the maintenance of central and peripheral tolerance. Their functionalized roles are; i) inhibiting memory and effector responses and, ii) inducing anergy, deletion, and tolerance of regulatory T cells (Treg) in the periphery, prior to the elimination of autoreactive T cells in the thymus through apoptosis (e.g., central tolerance) (Domogalla et al., 2017). Some studies had also emphasized the accessibility of tol-DCs to be developed from

matured DCs by stimulation with pro-inflammatory IFN-γ, apparently (Perry et al., 2014). Overall, DCs exhibit potential as favourable targets for immunotherapy as well as in allergic and autoimmunity.

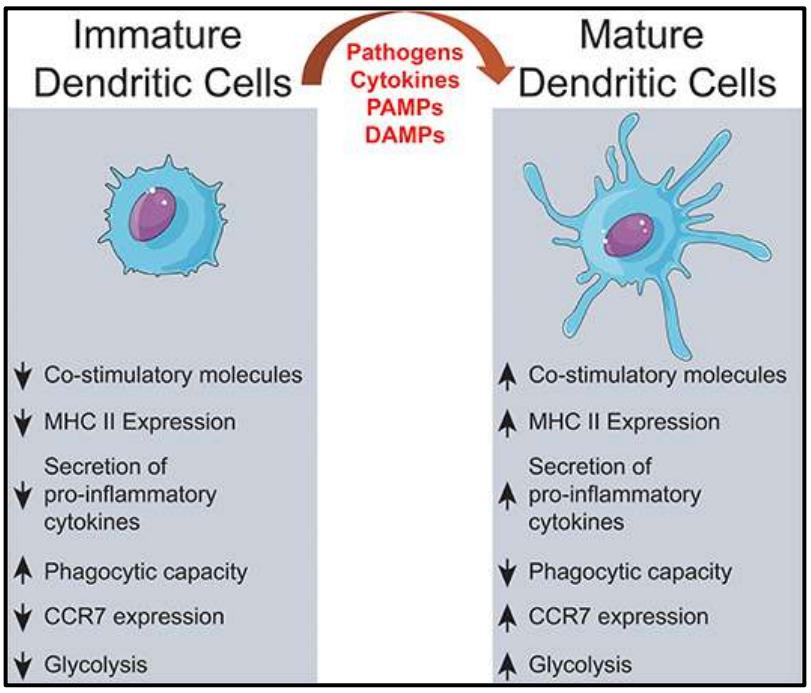

Figure 2.8 The formation of dendritic cells (Adapted from Patente et al., 2018)

2.3.1 Subsets of dendritic cells

In general, human DCs expressed high levels of HLA-DR and CD11c, with the absence of lineage-specific markers (Lin-1); CD3, CD14, CD16, CD19, CD56 markers (Collin & Bigley, 2018; Patente et al., 2018; Summers et al., 2001). There are three distinct classes of human DCs populations, such as myeloid DCs (mDCs), plasmacytoid DCs (pDCs), and monocytes-derived DCs (moDCs) (Figure 2.8) (Collin & Bigley, 2018). Each subtype of DCs is present with a distinctive proportion on different sites of the body. For instance, while mDCs are mostly accumulated in the lymphoid organs, pDCs are more likely to be present within the non-lymphoid tissues, and moDCs are found in the inflamed tissue (Chow et al., 2016). Specific

phenotypic markers and various genetic profiles are used to detect the subsets in each of the DCs populations.

2.3.1(a) Myeloid DCs

Myeloid DCs (mDCs) is defined as MHC $II^{+}CD11c^{+}CD123^{-}$, an innate immune cell activated upon encountering infection (Rhodes et al., 2019). The detection of Mtb pathogen via TLRs leads to the maturation of mDCs, which upregulate the cell surface molecule of MHC and other costimulatory molecules (Mendelson et al., 2006). It is mainly responsible for capturing, processing, and presenting the Mtb peptides to naïve T cells by migrating to the draining lymph nodes (Chistiakov et al., 2015). The mDCs are highly efficient in inducing primary T cell responses by exogenous and endogenous antigen presentation and secretions of cytokines (i.e., interleukin (IL)-7 and IL-10) (Liu, 2016). There are two major cell surface markers for the identification of human $CD11c^{+}$mDCs, namely CD1c and CD141 (Breton et al., 2016; MacDonald et al., 2002). The CD141 marker is mainly specialized in the cross-presentation of antigens to $CD8^{+}$ T cells, whereas CD1c is more likely to be involved in the presentation of lipid antigen to $CD4^{+}$ T cells (Haniffa et al., 2012). Both $CD1c^{+}$ and $CD141^{+}$ markers are designated to define mDCs subsets, $CD11c^{+}CD1c^{+}CD141^{-}$ (mDC1) and $CD11c^{+}CD1c^{-}CD141^{+}$ (mDC2), respectively (Wang et al., 2008).

The $CD1c^{+}$/BDCA-1^{+} (mDC2) is mainly used for $CD4^{+}$ biased-effector T helper cell responses (such as Th1, Th17, and Th2) against extracellular pathogens (Heger et al., 2020). It has been the most common surface marker for DCs within the PBMCs and is present in the blood and tissues (Schrøder et al., 2016). In an *in vitro* Mtb infection study, $CD1c^{+}$ showed higher responsiveness via TLR1-8 compared to $CD141^{+}$ and pDCs alongside the secretion of IL-6, TNF-α, and IL-1β cytokines, but

not IL-12p70 cytokine, upon the activation of CD4+ T cells (Lozza et al., 2014). This highlights the capability of $CD1c^+$ to promote various Th polarization *in vivo,* which are not induced by $CD141^+$ and pDCs (Liu et al., 2018). However, previous studies which obtained the blood of healthy individuals possessed a rather high level of IL-12p70 cytokine by the $CD1c^+$ (Leal Rojas et al., 2017; Nizzoli et al., 2013). Therefore, this current project will clarify further the secretion of IL-12p70 by comparing healthy individuals and TB conditions.

Meanwhile, $CD141^+$/BDCA-3^+ (mDC1) primarily specializes in antigen cross-presentation and promotes Th1 response via MHC I molecule (Breton et al., 2016; Tesfaye et al., 2019). This event leads to the activation of $CD8^+$ T cells and is required for an effective T-cell-based vaccine, instead of a direct $CD4^+$ T cell priming (Klechevsky, 2013). Human $CD141^+$ and $CD8\alpha^+$ DCs exhibit similar phenotypic characteristics such as TLR3, C-type lectin CLEC9A and novel surface molecule nectin-like protein l2 expression (Jongbloed et al., 2010; Pearson et al., 2018; Sabado et al., 2017). For instance, the splenic $CD11b^-$ $CD8\alpha^+$DCs and non-lymphoid tissue $CD11b^-CD103^+$DCs of murine model demonstrated high correlation with $CD141^+$DCs, emphasizing the variable vulnerability of various DCs populations against infection and PAMPs (Lozza et al., 2014). The percentage of $CD141^+$ is relatively rare with a low constitution of ~0.03% in human PBMCs (Jongbloed et al., 2010). $CD141^+$ has a lower secretion of IL-12 in comparison to $CD1c^+$ and mo-DCs, additionally (Collin & Bigley, 2018). Hence, $CD1c^+$ stimulates naïve $CD4^+$ T cells with high IL-12 secretion, meanwhile, $CD141^+$ acquires the dead cells for the subsequent cross-presentation of antigens to $CD8^+$ T cells.

2.3.1(b) Plasmacytoid DCs

Plasmacytoid DCs is a rare subtype of human DCs, which are derived from hematopoietic stem cells and recognized via CD123, CD303, and CD304 expression (Collin & Bigley, 2018; Li et al., 2017). The pDCs sense DNA and RNA viruses through TLR7/9, leading to the activation of pDCs and high secretion of IFN-I (Manz, 2018). A freshly isolated blood pDCs are less mature in comparison to mDCs and unable to stimulate the naïve T cells until being activated (Grouard et al., 1997). An activated pDCs upregulate both MHC class I and II with high expression of CD40, CD83, and CD86 costimulatory molecules, supporting its capability as an efficient APC (Jegalian et al., 2009; Li et al., 2017; Tel et al., 2012). Previous studies demonstrated high expression of IL-3 receptor α chain (CD123) with low CD4 and immunoglobulin-like transcript 1 (ILT1) expression by pDCs (Collin & Bigley, 2018). In general, pDCs majored in detecting and corresponding to active or inactivated viruses upon the intense production of IFN-I, IFN-*α,* and IFN-β, implicating the importance of pDCs in an early stage of viral infection (Mathan et al., 2013; Rogers et al., 2013). Although IFN-I is mostly involved as a potent anti-viral cytokine, it helps in the initiation of immune responses against Mtb by balancing the secretion of IL-12 and IFN-*α* cytokines (Lichtner et al., 2006). This event highlights the activation of mDCs by pDCs, which further induces T cells and Th1 polarization (Orsini et al., 2012). Besides, IFN-I promotes a protective role as a chemoattractant in TB infection. The mycobacterial-infected DCs mainly produce CXCL10 in response to IFN-I signaling, leading to the recruitment of immune cells to the site of infection in association to contain the spread of Mtb and stimulating effective immune response (Lichtner et al., 2006). However, human pDCs do not express lineage-specific markers or cytoplasmic immunoglobulin for the immune system

including CD11c, unlike mDCs (Cho et al., 2015). The gene transcription level strongly highlights no correlation between pDCs and activation of $CD4^+$ T cells in TB infection (Lozza et al., 2014). Consequently, $CD1c^+$ mDCs are the most highly responsive subset of DCs against Mtb infection with pDCs assisting $CD1c^+$ production.

2.3.1(c) Monocyte-derived DCs

The percentage of DCs in PBMCs which normally accounts for only 1-2% emphasize the importance of generating DCs from monocytes (Figueroa et al., 2016). Blood monocytes are the major origin of *ex vivo* DCs production (Coillard & Segura, 2019). The generation of moDCs has been commonly stimulated through the differentiation of monocytes alongside the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, and maturation medium (Sauter et al., 2019). The mo-DCs are primarily developed from $Ly6C^+$ inflammatory monocytes through a CCR-2-dependent mechanism and only are prominent upon inflammation (Chow et al., 2016). Most research and therapeutic applications are more likely to generate mo-DCs since they are readily available in comparion to the low percentage of mDCs (Sallusto & Lanzavecchia, 1994). In addition, the isolation of peripheral blood using $CD14^+$ monocytes or $CD34^+$ precursor cells are frequent in generating *in vitro* human mo-DCs (Chometon et al., 2020). In a previous study that used zeolite particles, the mo-DCs demonstrated a highly homogenous and selected population of DCs but with lower uptake and endocytic capacity in comparison to the freshly isolated mDCs (Chometon et al., 2020). Another recent study showed that the generation of mo-DCs with GM-CSF and IL-4 had rather produced a lower immune response (Dhodapkar et al., 2001). Although both mo-DCs and mDCs exhibited similar expression of HLA-DR, CD86, CD40, and CD83, yet different

morphological observations under light and electron microscopy have been reported (Osugi et al., 2002). The overnight cultured-blood $CD11c^+$ DCs illustrated higher cytoplasmic projections with a smaller number of dendrites compared to the larger size of mo-DCs which retained irregular cytoplasm and nucleus (Osugi et al., 2002). Therefore, mDCs have been chosen as the cell of interest in this study to explore in detail the uptake and activation of human DCs by liposomes.

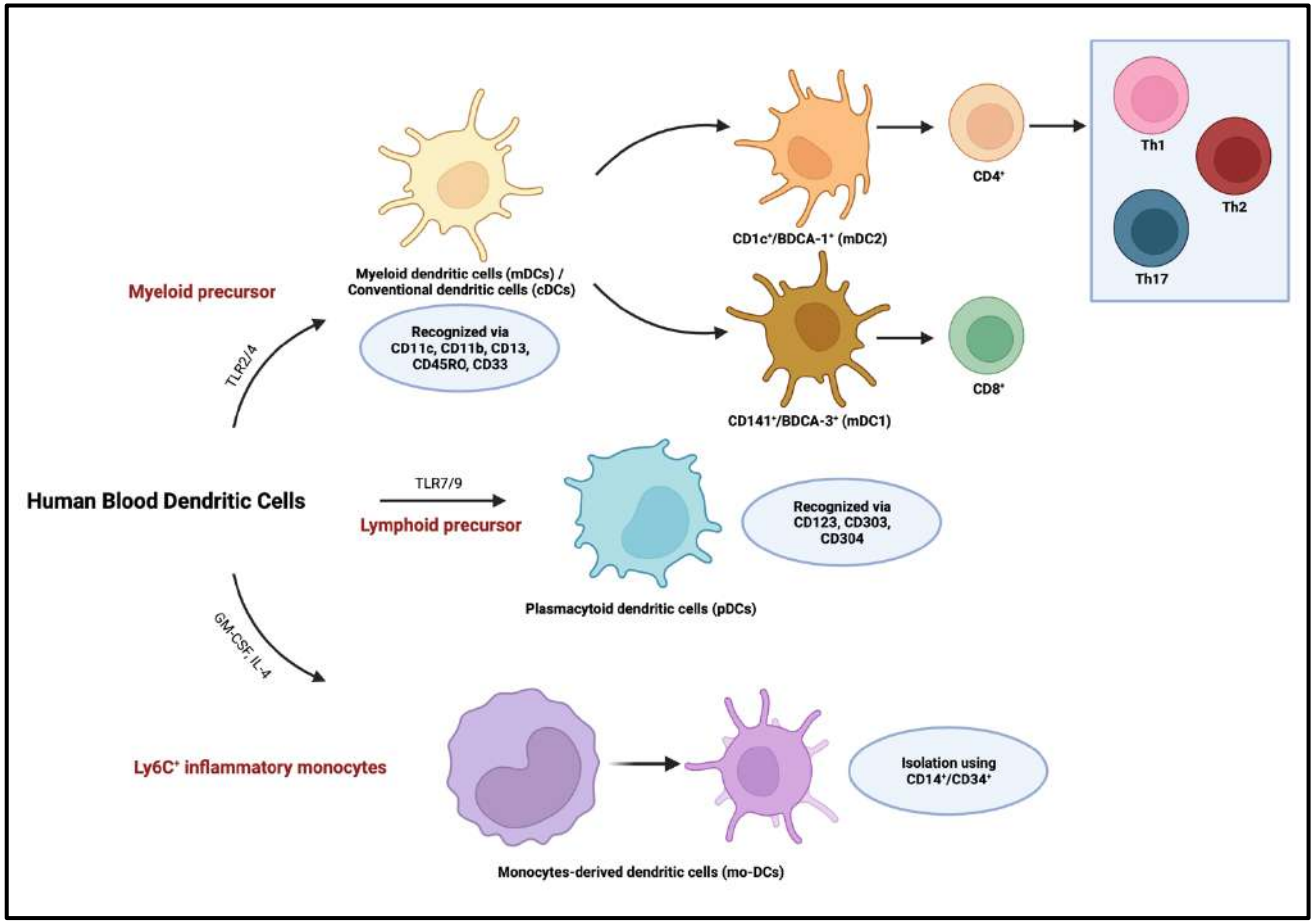

Figure 2.9 Subsets of human blood dendritic cells

There are three types of DCs subsets, namely, myeloid DCs, plasmacytoid DCs, and monocytes-derived DCs.

2.3.2 Uptake of antigens by dendritic cells

The presence of APCs is pivotal in priming effective immune responses against intracellular and extracellular pathogens (i.e., Mtb). Human DCs has been the most classical and accomplished APCs among immune cells, due to their robust ability to induce a primary immune response from the naïve T cells (Patente et al., 2018). There are numerous endocytic pathways involved during the initial uptake by immature DCs such as phagocytosis, receptor-mediated endocytosis, and micropinocytosis (Land, 2018). Similar to other phagocytic cells, DCs rely on their potent capacity to engulf Mtb pathogen for further intracellular processing (Land, 2018). The phagocytosis process demands PAMPs receptor recognition on the surface of Mtb (such as mannose receptor, complement receptor, DC-SIGN, scavenger receptor, or surface protein A receptor) via TLR signalling (Killick et al., 2013). For instance, the engagement of CD206 mannose receptor by Mtb on DCs has demonstrated a definite role of innate immunity in TB infection (Apostolopoulos & McKenzie, 2001; Naqvi & Endsley, 2020). CD206 is commonly expressed on immature DCs and macrophages and plays a crucial role in clearing the glycoproteins and inducing desirable immune responses (Garcia-Aguilar et al., 2016; Suzuki et al., 2018). Previous studies showed the induction of a downstream signalling response which assists in Mtb elimination via the phagocytosis process (Dorhoi et al., 2010). This condition occurs through CARD9 ligand binding of type II transmembrane CLR, Dectin-1, which is commonly expressed on myeloid cells including DCs (Wagener et al., 2018). The association of DCs in primary non-MHC-restricted and CD1-restricted T cells through lipoglycan lipoarabinomannan (LAM) has also been discussed (Tailleux et al., 2003). The intracellular trafficking of DC-SIGN in Mtb-infected DCs exhibited its participation in transporting the Mtb

glycolipids from the vacuole to the cell membrane (Ernst et al., 1998). These events highlight the uptake of Mtb proteins by human DCs, allowing the digestion of enzymatic pathogens via phagolysosomes (phagosomes fused with MHC-II lysosomes) (Land, 2018).

2.3.3 Activation of dendritic cells

Human DCs are present in most tissues, performing as a link between innate and adaptive immune responses. They can be found in two different functional states, 'immature' and 'mature', with contrasting morphology and phenotypic features (Patente et al., 2018). The surface of immature DCs is highly described as circular and even, which is contradictory to the mature DCs of having rough and numerous pseudopodia (Kim & Kim, 2019). Immature DCs are poor stimulators of effector T cells due to the low expression of co-stimulatory molecules and chemokines expressed, however, they are more likely to be efficient during the uptake of Mtb via TLR signalling (Patente et al., 2018). This event indeed highlights immature DCs as the 'sentinels' upon Mtb infection and 'tissue scavengers' of necrotic and apoptosis cells (Albert et al., 1998). The interaction between immature DCs with Mtb pathogen leads to the activation of mature DCs, stimulating T cells response (Dudek et al., 2013). The activation of antigen-specific T cells in secondary lymphoid organs mainly highlights the hallmark of mature DCs (De Santis et al., 2019; Hawiger et al., 2001). Matured DCs may have low endocytic activity but demonstrate a high motility rate with upregulation of MHC-II, co-stimulatory molecules, cytokines, and chemokine receptor expression (Reis e Sousa, 2006). For instance, mature DCs in TB infection exhibit rather low endocytosis, phagocytosis, CCR1, CCR5, and CCR6 levels, yet express a high surface level of MHC-II, CD86, CD80, CD54, CD58, CD83, CCR7, and CD40 (Banchereau et al., 2000).

Mycobacterial components have shown their engagement in innate recognition and responses via the TLR pathway (Byun, Kim, Kim, et al., 2012). For instance, Mtb antigens (i.e., Rv1196, Rv0978c, and Rv0754) induce the maturation and activation of DCs through TLR2-dependent signalling (Bansal et al., 2010; Byun, Kim, Shin, et al., 2012). This event leads to the secretion of proinflammatory cytokines such as IL-12p70, IL-17, IFN-γ, and TNF-α, which further produce Th1 and Th17 responses (Lyadova & Panteleev, 2015). The central role of the Th1 cell in human TB occurs through IFN-γ secretion, leading to the activation of macrophages and Mtb restriction via phagocytosis (Weiss & Schaible, 2015). The induction of IL-12p70 by DCs enhances $CXCR3^{+}CCR5^{+}$ to send a signal to Th1 and stimulates IFN-γ, which further transmits DCs signalling to secrete higher level of IL-12p70 (Saha et al., 2013). There have been conflicting opinions on the level of Th1/IFN-γ in both latent TB infection and active pulmonary TB patient, yet the differences in IFN-γ response is unvarying. For instance, the stimulation of Th1/IFN-γ response could either be strong or weak in both latent TB infection (LTBI) individual and active pulmonary TB patients due to the differences in genetic host background, the virulence of infected strains, and their immune levels (Lyadova & Panteleev, 2015). These highlighted Th1-mediated IFN-γ response not only contributes to the elimination of the Mtb but also during the mid-treatment and post-treatment of TB infection (Lyadova & Panteleev, 2015). The participation of Th17 cells in TB pathogenesis has been observed through the induction of neutrophil inflammation and mediation of tissue damage, which is quite complicated (Lyadova & Panteleev, 2015; Shen & Chen, 2018). Th17 development is dependent upon IL-23 secretion in the presence of low TGF-β (Khader & Cooper, 2008). Mtb induces DCs to secrete IL-23 cytokine which promotes the activation of Th17 cells, leading to the secretion

of IL-17 *in vitro* (Shen & Chen, 2018; Yang et al., 2006). Both IL-17 and IFN-γ are Th17-related cytokines that encourage the recruitment of cells and granuloma formation in TB infection (Torrado & Cooper, 2010). The IL-23/IL-17 had demonstrated their pivotal role as an efficient modulator of immune response in all stages of Mtb infection by triggering chemokines to recruit $CD4^+$ T cells, which ultimately inhibit the survival of Mtb (Khader et al., 2007). Hence, Th17 cells exhibit IFN-γ independent protection against TB and assist in controlling TB disease (Wozniak et al., 2010).

2.4 Cytokines related to tuberculosis

Cytokines are known as small soluble secreted proteins (~5-20 kDa) released by diverse cell types, which functionalized to mediate immune and inflammatory responses through the communication between cells (Domingo-Gonzalez et al., 2016). The release of cytokines into blood circulation or tissues allows the immune cells to locate and bind to their cell-assigned receptors (Khan, 2016). Different chemical signalling by cytokines can either occur in the production site (autocrine effect), in neighbouring cells (paracrine effect), or in distant cells (endocrine effect) (Jang et al., 2015). They are highly crucial for immune protection against foreign pathogens. For instance, DCs maturation is an essential process to present efficient Mtb antigen for the stimulation of proper immune responses (Liu et al., 2017; Prendergast & Kirman, 2013). Therefore, various cytokines with pro-inflammatory and anti-inflammatory effects are produced by DCs in response to the induction of Th1, Th2, or Th17 during Mtb infection.

2.4.1 IFN-γ

The IFN-γ cytokine has been acknowledged for its pleiotropy characteristic in priming innate and adaptive responses upon exposure to intracellular pathogens, specifically in TB infection (Lu et al., 2019; Schierloh et al., 2007). It is usually released by $CD4^+$ T cells, specifically Th1 cells upon the activation of DCs. The early release of IFN-γ assist in the activation of macrophages, which in turn become more efficient at containing and controlling TB infection (Cavalcanti et al., 2012; Robinson et al., 2010). It has been known as a homodimer developed from two 17 kDa non-covalent polypeptide subunits and exists as the only member in type II interferon that binds to IFN-γ receptor (IFNGR) (Castro et al., 2018; Zha et al., 2017). Previous studies demonstrated an enhanced susceptibility to Mtb in mice models and human clinical trials with deficient genes and receptor of IFN-γ (Lalvani & Millington, 2008; Ottenhoff et al., 1998). IL-12 is the most notable cytokine-stimulated by DCs, serving as a bridge to connect TB infection with IFN-γ production, via triggering of TLR2 (Crow et al., 2012; Fricke et al., 2006; Schroder et al., 2004). The classical pathway demonstrated the secretion of either IFN-γ and IL-12 or IL-12 alone by $CD4^+$ T cells via Th1 response (Lalvani & Millington, 2008). For instance, the secretion of IFN-γ cytokine in TB leads to the activation of mycobacterial mechanisms in macrophages and cytotoxic T cells that further restrict the growth and dissemination of Mtb (Fan et al., 2012). The previous finding also demonstrated that IFN-γ response is more predominant in active pulmonary TB patients compared to LTBI individuals, suggesting strong association between IFN-γ cytokine with TB activity (Nikitina et al., 2016).

2.4.2 IL-12p70

The family group IL-12 plays a major role in secreting IFN-γ that contributes to the activation of $CD4^+$ T cells and differentiation into Th1 cells, initiating the desired immune responses (Gee et al., 2009; Méndez-Samperio, 2010; Sun et al., 2015). The IL-12 cytokine is predominantly produced by APCs such as DCs and macrophages (Urazova et al., 2019). Meanwhile, IL-12p70 cytokine which is part of the IL-12 family, mainly comprises p35 and p40 subunits and its secretion could be enhanced by IL-27 cytokine. It has been demonstrated that both IL-12p70 and IL-27 contribute in stimulating Th1 immune response (Méndez-Samperio, 2010). For instance, human DCs had shown IL-12p70 secretion upon Mtb exposure, which leads to $CD4^+$ T cells differentiation into Th1 response (Frasca et al., 2008). This event allows IFN-γ production, initiating a positive feedback loop for signal DCs to continuously potentiate IL-12p70 and Th1 immunity (Abrahem et al., 2020; Muller-Berghaus et al., 2005). The association between IL-12p70 by mDCs and IL12B gene polymorphism also highlights its role in the growth of infiltrative and disseminated human pulmonary TB (Urazova et al., 2019). Furthermore, *in vivo* studies supported through high secretion of IL-12 cytokine in response to mononuclear phagocytes to Mtb infection, activating $CD4^+$ T cells that lead to the elimination of Mtb in tuberculous pleuritis patients (Cooper & Khader, 2008; Feng et al., 2005; Flynn et al., 1995; Fulton et al., 1996). These events proved the essential function of IL-12p70 in TB infection.

2.4.3 IL-4

A single Th1 response does not guarantee sufficient protection in Mtb, leading to a subversive Th2 response in the Th1 environment (Pooran et al., 2019). IL-4 is an anti-inflammatory cytokine that orchestrates Th2 immunity, however, its

pivotal role has been controversial in TB disease (Lazarski et al., 2013; Pooran et al., 2019). It has been an important cytokine in the immune responses to TB, which contributes to DCs formation via the differentiation of monocytes (Bai et al., 2004). In previous studies, active pulmonary TB patients secreted a remarkable increase in IL-4 cytokine through $CD4^+$ and $CD8^+$ T cells, antagonizing host defense that leads to the tissue necrosis in comparison to healthy individuals (Bai et al., 2004; van Crevel et al., 2000). Conversely, the differential expression of IL-4 levels and failure to discriminate between IL-4 and the IL-4 splice variant, IL4δ2, have been reported (Djoba Siawaya et al., 2008). The IL-4 could be functionalized as a negative regulator by suppressing IL-12 signalling, leading to the inhibition of $CD4^+$ T cells differentiation into Th1 or Th17 cells (Harrington et al., 2006). For instance, a ~50% reduction in Mtb containment has been observed upon IL-4 secretion in human TB (Semple et al., 2013). Similarly, IL-4 showed a significant TB susceptibility in a different study, supporting its deleterious effect on human extrapulmonary and severe TB conditions (He et al., 2018; Qi et al., 2014). It has been demonstrated that patients with TB secrete high IL-4 with increased mRNA level, IL4δ2, emphasizing the need for efficient vaccines to target Th2 suppression and impaired the Mtb function (Rook et al., 2004). This study will be focusing on IFN-γ, IL-12p70, and IL-4 responses upon the exposure to liposomes in TST-negative individuals, TST-positive individuals, and active pulmonary TB patients.

2.5 Liposomes

Liposomes are nano-spherical vesicles comprised of a lipidic bilayer with a close resemblance to the structure of a cell membrane (Cheepsattayakorn & Cheepsattayakorn, 2013; Rai et al., 2015). The term originated from two Greek

words, with “Lipo” expressing fat and “Soma” indicating body or structure (Sharma & Agrawal, 2021). The liposomes were primarily discovered by a British haematologist Alec D. Bangham in 1961, and further demonstrated by Gregoriadis G. (1972) as a potential delivery carrier in therapeutics applications (Gregoriadis, 2016). The significant roles of liposomes in immunology have been examined through gene delivery, antiviral therapy as well as in the delivery of drugs and proteins actions (Nisini et al., 2018). Interesting descriptions of liposomes will be further discussed in the context below.

2.5.1 Unique features of liposomes

There are a few main parameters highlighting liposomes as the most efficient delivery system. Liposomes have the unique capability of encapsulating a diverse type of hydrophilic and hydrophobic drugs in their aqueous centre (Figure 2.9) (Zhang et al., 2017). The polar heads of liposomes orient towards the aqueous medium with the hydrophobic tails self-assemble into the inner region, structurally (Nagalingam, 2017). These properties allow the internal hydrophilic part to protect against degradation of the loaded drugs by minimizing the adverse effects while altering the pharmacokinetic activity of drugs upon being embedded into the lipid membranes (Alavi et al., 2017; Nakhaei et al., 2021). Liposomal encapsulation of drug has been well studied for possessing a limited toxicity effect with an absence in pyrogenic or antigenic reactions (Singh & Goyal, 2013).

In addition, the liposomal formulation enhances an efficient delivery of therapeutic drugs due to its phospholipid composition, which is biologically inert, feebly immunogenic, and derived from natural sources (Çağdaş et al., 2014). For instance, unsaturated phosphatidylcholine such as soybean and egg sources, rather demonstrated a highly permeable with less stabile bilayers, which is contradictory to

the rigid and impermeable structure of dipalmitoyl saturated phosphatidylcholine bilayer (Akbarzadeh et al., 2013). In a previous study that loaded rifampicin into freeze-dried soy lecithin liposomes, a rapid, prolonged, and high efficiency of encapsulated rifampicin to macrophages supported the potential of liposomes as a successful therapy for TB (Patil et al., 2015). The interaction between liposomal lipid derived from *Mycobacterium smegmatis* with murine bone marrow DCs further acknowledged the immune activation of liposomes (Mat Luwi et al., 2020).

Liposomes could provide potent targeting approaches for TB therapy through both active and passive delivery ligands, additionally (Quijia & Chorilli, 2022). A rifampicin-loaded mannosylated and polyethylene glycol (PEG)-ylated graphene oxide liposomes facilitated an efficient uptake of the macrophages via active targeting, which further leads to a competent inhibition *in vitro* and *ex vivo* of intracellular Mtb (Mazlan et al., 2021). This proves that active targeting by liposomes enhances specific, selective targeting in pulmonary TB (Pinheiro et al., 2011). Plenty of studies exhibited significant liposomal achievements through passive targeting, such as amikacin-SUVs liposomes which demonstrated an increased activity against Mtb with a prolonged biological half-life (Dhillon et al., 2001). Passive targeting in pulmonary TB in combination with intravenous and inhalation routes has received lots of attention due to the small sizes of liposomes that can be easily taken up by phagocytic cells such as macrophages and DCs (El-Ridy et al., 2007). In general, versatility and plasticity remained the major strengths of liposomes. A variety of physicochemical and biophysical characteristics for selecting the desirable composition of liposomes may include particle size, lipid types, charge, etc. (Schwendener, 2014; Sercombe et al., 2015). Furthermore, the uncomplicated preparation of liposomes eventually helped to ameliorate a new novel

platform technology (Chen et al., 2012). These factors had pointed out liposomes as an interactive carrier for drug delivery (Christian I. Nkanga et al., 2019). Therefore, it is of great interest to analyse the utilization of liposomes to be used as a significant attractive vehicle for the improvement of drug or vaccine delivery systems against targeted diseases (Daraee et al., 2016).

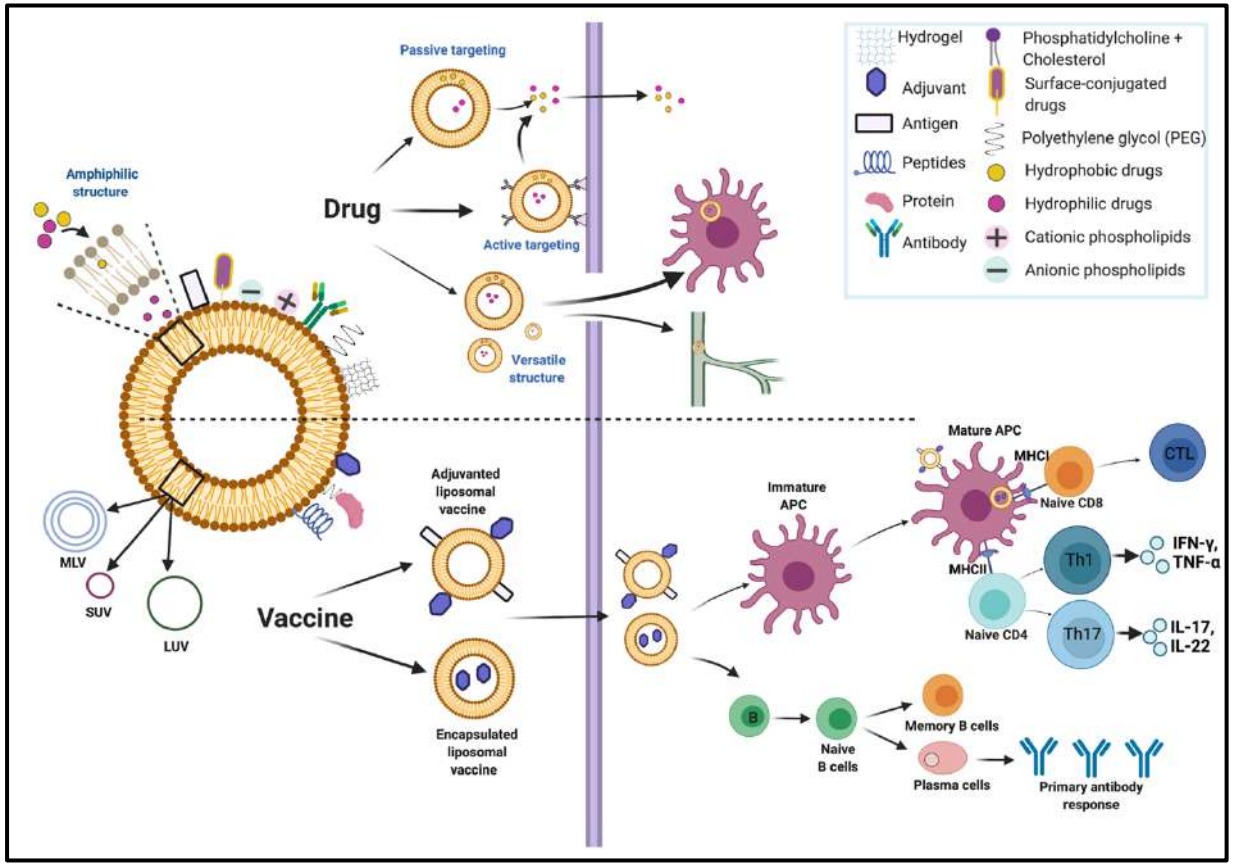

Figure 2.10 Advantages of liposomes as drug delivery and adjuvant (Adapted from Suhaimi et al., 2022)

The unique properties of liposomes highlighted their pivotal role as the efficient carrier and adjuvant in the drug delivery system and vaccine development, respectively.

2.5.2 Classification of liposomes

The major factors that contribute to liposomal production are dimensions, the number of bilayer membranes, and phospholipid charges (Pamunuwa et al., 2016). They are mainly responsible for determining the volume of drugs needed to be encapsulated into the liposomes. There are various formations of liposomes that can be found; from small-scale up to massive proportions, with a usual ranging sizes between 0.025 μm to 2.5 μm (Sakuragi et al., 2011). The sizes play a critical role in regulating the half-life circulation of liposomes. On that account, liposomes can be further classified into two main groups, namely unilamellar vesicles and multilamellar vesicles (MLVs) (Akbarzadeh et al., 2013). Unilamellar liposomes mainly refer to spherical vesicles surrounded by a single phospholipid bilayer, contrary to multilamellar liposomes which consist of numerous layers of concentric lipids with higher particle sizes (Khan et al., 2013). Both are efficient in their ways, in which unilamellar has a higher internal core volume to confine hydrophilic molecules (water-soluble drugs), meanwhile, MLV is solely focused on incorporating hydrophobic molecules, due to its intense lipid content (Azanza et al., 2015; Chaves et al., 2018).

The liposomes have been recently categorized concerning their functions such as conventional liposomes, ligand-targeted liposomes, long-circulating stealth liposomes, and multi-functional liposomes (Patil & Jadhav, 2014). Conventional liposomes are the pioneer generation of liposomes (X. Wang et al., 2016). It possesses various types of lipid composition, with phosphatidylcholines and cholesterol being named as its most flexible lipids (Sercombe et al., 2015). Previous *in vivo* studies had demonstrated conventional liposomes to minimize the toxicity levels in compounds by altering the biodistribution and pharmacokinetics of

encapsulated drugs (Sercombe et al., 2015). However, it had shown restriction through bloodstream swift rejection (Sercombe et al., 2015). Meanwhile, ligand-targeted liposomes offer a site-specific drug delivery to particular organs or cells *in vivo* (Noble et al., 2014). Specific ligands (i.e., antibodies, receptors, cell adhesion molecules) are activated at the site of diseases upon binding to the surface of liposomes, thus forming immunoliposomes (Eloy et al., 2017). Optimum substrate communication between surface-coupled ligands and liposomes has been highly reported (Puri et al., 2009). Nevertheless, the low efficiency of immunoliposomes is the major drawback confronted as a drug carrier (Puri et al., 2009). Therefore, long-circulating liposomes overcome the limitations of the earlier generation of liposomes as another alternative approach (Deodhar & Dash, 2018). Coating with poly-(ethylene glycol) (PEG) or stealth liposomes is one of the highly anticipated liposomal production (Immordino et al., 2006). This is mainly achieved by the occupancy of PEG derivatives onto the outer membrane of the liposome, which reduces the uptake of the mononuclear phagocyte system (MPS) and elongates the duration of circulating blood (Bangale et al., 2014). Yet, it has the potential to serve as a prolonged reservoir of therapeutic agents (Immordino et al., 2006). Therefore, multifunctional liposomes have been further developed as the latest generation of liposomes (Aryasomayajula et al., 2017). This newly designated platform is an improved fundamental synthesis of previously discussed liposomes by the efficient response to the external and internal stimuli such as pH, enzyme level, temperature, and penetration into the cells (Aryasomayajula et al., 2017). In general, liposomes have succeeded to evolve competently as one of the powerful platforms for delivering drugs to numerous diseases.

2.5.3 Sources of phospholipids in liposomes

Composition is one of the contributing factors that categorized liposomes. The root of phospholipid choices mainly regulates the permeability and charges of the bilayer, in which they could be utilized from either natural or synthetics derivatives (van Hoogevest & Wendel, 2014).

2.5.3(a) Liposomes derived from non-mycobacteria

The discovery of complex aliphatic compounds was initially demonstrated by Fourcroy (1793), followed by the finding of human brain phospholipid in 1812, and a successful attempt to separate phospholipids from the egg yolk by Gobley (1846) (Li et al., 2015). Natural sources such as soybean, egg yolk, and sunflower seed are the most extracted phospholipids, due to lower and cheaper costs in comparison to synthetic methods (Akbarzadeh et al., 2013). In previous studies, soybean lecithin has the advantages of being stable, safer, and abundantly available in purified and non-purified components at a reasonable cost (Le et al., 2019). Yet, lecithin from sources other than soybean exhibited a low stability effect due to the high concentration of polyunsaturated fatty acids and has a higher potential for protein contamination (Miranda et al., 2015). On the contrary, researchers would highly prefer to use synthetic phospholipids due to the simplified single defined structure components (Li et al., 2015). For instance, the incorporation of synthetic amphiphiles with sterols such as cholesterol has been well established in multiple areas (Nakhaei et al., 2021). Overall, both sources of liposomes are widely used in the pharmaceutical fields as drug carriers (Bulbake et al., 2017). In the meantime, other sources have been investigated and tested for their suitability and potential for the development of liposomes.

2.5.3(b) Liposomes derived from mycobacterium

Live-attenuated mycobacterium strain targeting lipids has been recently studied as one of the high potential vaccine candidates. Lipid, the constituents of the waxy mycobacterial cell wall is the most suitable target for immune defense, due to their potential role against the resistance of pathogen in TB disease (de Pablo et al., 2000; Ciamak Ghazaei, 2018). In addition, lipids showed distinctive critical roles in the cell physiology such as regulating signalling, intracellular vesicle trafficking, and phagocytosis mechanism (Nisini et al., 2018). The mycobacterial cell envelope is complex and comprises numerous lipids and polymers which modulate the activation and differentiation of cells and influence Mtb pathogenicity (Pouget et al., 2021). These molecules are localized at the cell surface and part of the mycobacterial cell wall, highlighting them as a potential target for host immunity (Morandi et al., 2013). For instance, a lipid extract from *Mycobacterium bovis* BCG conjugated with apolar single specific lipids, monomycoloyl glycerol (MMG) showed a prominent Th-1 biased immune response (C. A. Andersen et al., 2009). In a different study, similar outcomes of strong antigen-specific immune responses with high expression of IFN-γ and antibodies supported the immunostimulatory effect of total lipid extract of *Mycobacterium bovis* BCG (Rosenkrands et al., 2005). Likewise, liposomes formulation composed of natural lipid extraction derived from glycolipids of several different types of bacteria such as *M. smegmatis, E. coli, Neisseria meningitidis,* and *Leptospira biflexa serovar Potac* has been developed. The protective effect against Mtb enhanced by formulated liposomes derived from Mtb lipids conferred both specific humoral and cellular immune responses in a guinea pig model (Dascher et al., 2003; Singh & Khuller, 1993b, 1994). These studies highlighted the excellent uptake and activation of APCs (mainly by DCs) with an improved localization in the

draining lymph nodes by liposomes. In addition, a significant reduction in the bacterial load exhibited by lipid-based Ms of alum-adjuvanted and nonadjuvanted formulations demonstrated similar outcomes to the BCG group (García Mde et al., 2014; Sorokoumova et al., 2009). Hence, liposomes are highly correlated with the enhancement of specific and cellular immune responses and possess immunoadjuvant capacity in experimental mice models (Faisal et al., 2011).

2.5.4 Method of liposomes production

The method of preparation also divides the liposomes, ultimately. There are two major methods used to encapsulate drugs into liposomes, namely, passive loading and active loading (Pauli et al., 2019). Passive loading method is a process of consigning entrapped agents and implemented either before or during the preparation process. Meanwhile, delivering compounds that are ionizable and highly soluble in both water and lipids, to liposomes after the induction of intact vesicles, is called the active loading method or “remote loading” (Dua et al., 2012). The ideal formulation of liposomes is highly required to obtain efficient drug entrapment and maintain its stability in the long term. Various strategies are performed to complete the liposomal preparation such as mechanical methods using thin-film and ultrasonic methods (e.g., solvent dispersion approach, the fusion of preformed vesicles, or transformation of sizes).

Mechanical dispersion has been the most recurring method due to its simplicity, which can be performed in various ways (i.e., freeze-drying, hand-shaking, non-hand-shaking, etc. (Powers & Nosoudi, 2019). It is outlined by thin lipid-film hydration (known as the Bangham method), which resulted in a heterogeneous population of multilamellar vesicles (MLVs) that can be further sonicated to induce small unilamellar vesicles (SUVs) (Christian Isalomboto Nkanga

et al., 2019). Another adopted preparative alternative implying film hydration is the reverse-phase evaporation vesicles (REV) using a solvent dispersion strategy (Nele et al., 2019). Inverted micelles or water-in-oil emulsions are formed within the aqueous phase (consisting of hydrophilic materials) and organic phase (consist of hydrophilic materials and lipids) upon sonication (Wagner & Vorauer-Uhl, 2011). This method is more proficient in loading a greater number of both small molecules and macromolecules into the internal aqueous core (Shi & Qi, 2017).

Methods based on the fusion of preformed vesicles or size transformation are part of the preparation of liposomes. It consists of two approaches: the freeze-thaw extrusion method and the dehydration-rehydration method (Figure 2.10) (Prathyusha et al., 2013). In general, the freeze-thaw extrusion technique is mainly resulting in liposomes of MLVs. The liposomes are prepared by film method and vortexed to entrap the solute until the whole film is fully suspended. Six cycles of freeze-thawing processes and an additional 8 extrusions are required for obtaining the final product of liposomes. Conversely, the dehydration-rehydration method is a simple process involving mixing and drying of empty buffers (consisting of SUVs) with the components used for entrapping which are known as lyophilization. Conventionally, dehydration-rehydration method is the preferable option. Solid lipids are dispersed into ultra-fine formation, and the vesicle is rehydrated. In the end, liposomes derived are usually in oligo lamellar vesicles. This is mainly due to the fusion of concentrated vesicles upon dehydration and stacking of solutes in multilamellar planes (Rahman et al., 2018).

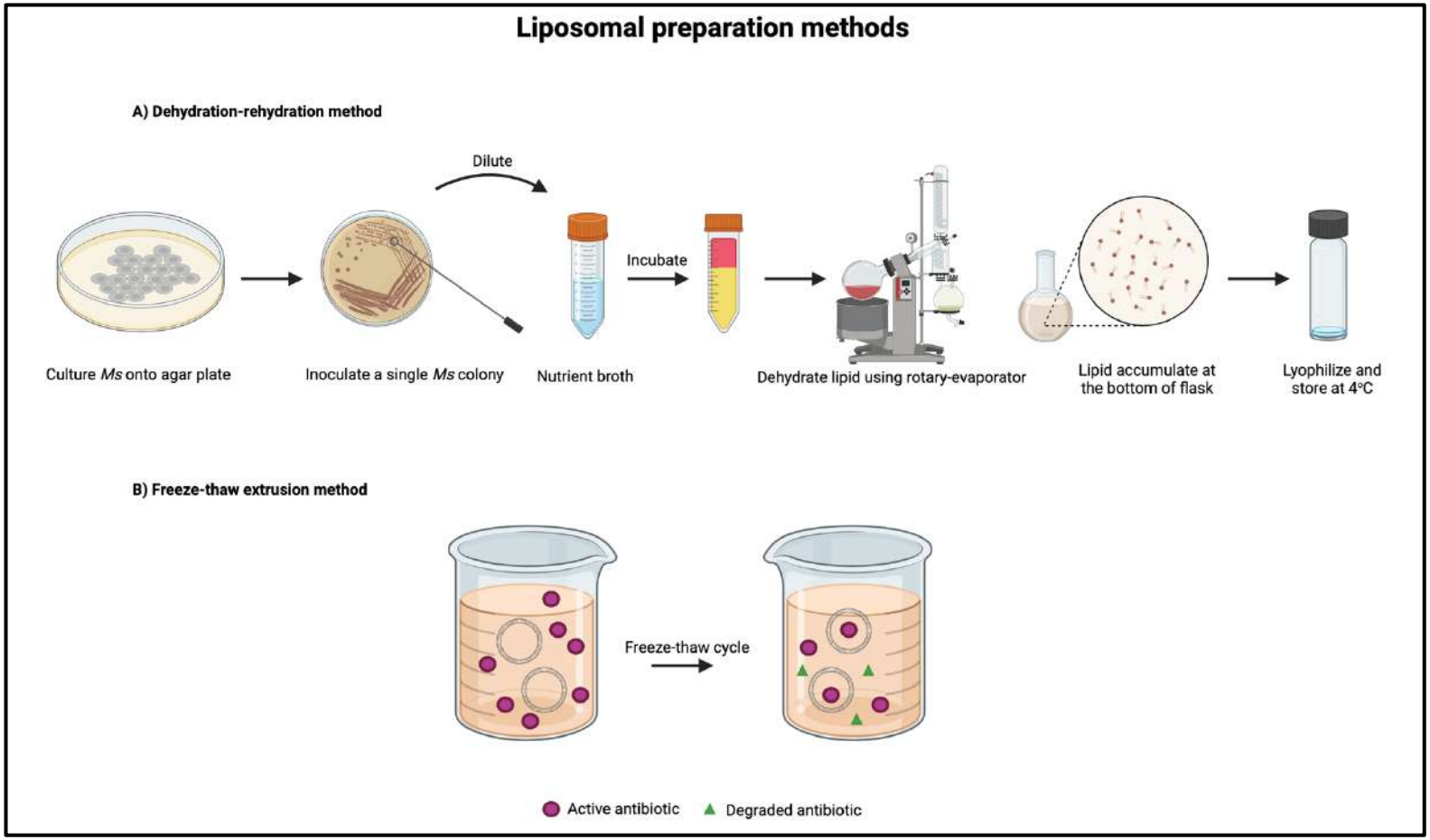

Figure 2.11 Method of producing liposomes

The figure above represents the two main methods in producing liposomes, namely dehydration-rehydration and freeze-thaw extrusion.

2.5.5 The use of liposomes in biomedicine

The flexible properties of liposomes have improved the challenges in various applications either in the drug delivery system or vaccine adjuvant. Liposomes are proposed to provide optimum efficacy with the uniform circulation of the drugs to the target site while reducing the side effects (Ibrahim Bekraki, 2020). The encapsulation of conventional drug therapy by liposomes stabilizes both hydrophilic and hydrophobic drugs through the enhanced solubility and permeability of anti-TB drugs (Li et al., 2020). In TB infection, liposomes formulated with isoniazid, rifampicin, and pyrazinamide showed an increased relative bioavailability and high *in vitro* drug release in comparison to the unencapsulated drugs (Suhaimi et al., 2022). The formulation of two coumaran (2,3-dihydrobenzofuran) derivatives—TB501 into PEGylated liposomes demonstrated vesicular stabilization with good nontoxicity and homogeneity effects of the antitubercular drugs (Kósa et al., 2021). In addition, liposomes also can prolong the retention period of entrapped drugs within the infected cells, facilitating the intracellular delivery of anti-cancer drugs (Allahou et al., 2021). Doxil, the first liposomes formulation that comprises doxorubicin drug, exhibited minimized toxicity levels with efficient treatment against cancer (Zhang et al., 2021).

Vaccination has been proven to stimulate the immune system of the body and fight against foreign pathogens such as viruses or bacteria, thereby inhibiting the complications of diseases (Pollard & Bijker, 2021). The fundamentals of the vaccine have been initially discovered through a smallpox vaccine development, particularly used to resist the eradication of fatal viruses during the late eighteenth century (Riedel, 2005). There are various types of vaccine adjuvants, a substance with intrinsic immunomodulatory characteristics which potentiate the host antigen-

specific immune responses effectively, including liposomes (Wang et al., 2019). The usage of liposomes as vaccine adjuvant was initially demonstrated in 1974 through a mice model injection of phospholipid-based liposomes which was adjuvanted with diphtheria toxoid (DT) (Allison & Gregoriadis, 1974). Higher titers of antibody were obtained in comparison to the non-adjuvanted DT, thus leading to further detailed continuous studies and clinical trials (Schwendener, 2014). Virosomes are known as one of the active fields studied, which involves the revision of current vaccines by liposomes addition or the development of new liposomal adjuvants vaccines. Various applications such as natural or synthetic phospholipids liposomes, non-phospholipid cationic liposomes as well as combined immunostimulants-liposomes have been discovered (Müller & Landfester, 2015).

BCG vaccination remains the only available vaccine against TB infection with limited efficacy protection in adults (Ottenhoff & Kaufmann, 2012). This main issue urged the need for a newly developed vaccine, in which liposomes shined as a competent adjuvant. In a previous finding, novel liposomes CAF01 adjuvanted with a TB vaccine Ag85B-ESAT-6 (H1) demonstrated a rather safe, well-tolerated with enduring T cell responses in a human clinical trial (van Dissel et al., 2014). This study has been supported by a liposomal adjuvant dimethyldioctadecylammonium (DMT) emulsified with CMFO protein, which exhibited similar immunization protection against primary infection, latent infection, and reactivation of TB disease in a mice model (Ma et al., 2017). In a further investigation, the CFMO/DMT liposomes possessed a higher synergistic effect, durable and stronger lung protections with increased levels of IFN-γ, TNF-α, IL-17A, and IL-2 in comparison to the CMFO liposomes emulsified with DDA or DDA/TDB adjuvants (Hao et al., 2020). Furthermore, the development of cationic liposomes-based vaccines has been

widely studied due to their competency to prime strong humoral and cellular immune responses against TB disease (F. Khademi et al., 2018). In addition, these cationic liposomes serve as an efficient adjuvant and delivery carrier by inducing a strong memory response, potent electrostatic interaction with APCs, and prolonged the storage of subunit TB vaccines at the site of injection (F. Khademi et al., 2018). However, toxicity is the major drawback of the cationic liposomes, leading to their combination with other immunostimulatory components (Cui et al., 2018).

CHAPTER 3

METHODOLOGY

This section describes in detail the study design, study area, study duration, and study sampling. The materials used throughout the whole study are elaborated. Specific methods, as well as data and sample collection, were further illustrated in each respective chapter.

3.1 Antibodies

Fluorochrome-labelled antibodies used in this study are listed in Table 3.1.

Table 3.1 Fluorochrome-labelled antibodies run in flow cytometry

Antibody	Fluorochrome	Company
CD11c	APC anti-human	Biologend, USA
HLA-DR	APC/Fire anti-human	Biologend, USA
CD123	PE anti-human	Biologend, USA
CD86	PE/Cy7 anti-human	Biologend, USA
Viability staining solution	7-AAD	Biologend, USA

3.2 Chemicals and reagents

General chemicals and reagents used in this study are listed in Table 3.2.

Table 3.2 Chemicals and reagents

Chemicals/Reagents	Company
2-propanol	Merck, Germany
Carbol fuchsin	Merck, Germany
Chloroform	BDH Chemicals, UK
Coomassie Brilliant Blue	Sigma Aldrich, USA
Ethanol absolute (C_2H_5OH)	BDH Chemicals, UK
Fetal Bovine Serum (FBS)	Capricorn Scientific, Germany
FITC antibody	Thermo Fisher Scientific, USA
Glycerol	Riedel de Haen, Germany
Hoechst stain 33342	Tocris Bioscience, UK
Lipopolysaccharides (LPS)	Thermo Fisher Scientific, USA
Lymphoprep	Stemcell, Germany
Methylene blue	Merck, Germany
Nutrient agar	Merck, Germany
Nutrient broth	Merck, Germany
Phalloidin-Rhodamine dye	Abcam, UK
Phosphate-buffered saline (PBS)	1st BASE Biochemicals, Singapore
Roswell Park Memorial Institute (RPMI 1640 with glutamine and histamine)	Thermo Fisher Scientific, USA
Sodium hydroxide (NaOH)	HmbG Chemicals, Germany
Sodium sulphate (Na_2SO_4)	R&M Chemicals, UK
Tween 80	Sigma Aldrich, USA
Yeast extract	Oxoid, UK

3.3 Laboratory equipment and apparatus

All laboratory equipment used in completing this project are as listed in Table 3.3.

Table 3.3 Laboratory equipment and apparatus

Laboratory equipment/Apparatus	Company
Balance	Mettler Toledo, Columbia
Biosafety cabinet Class II	ERLA Technologies, Malaysia
Centrifuge 4000	Kubota, Japan
Centrifuge 5810 R	Eppendorf, Germany
Carbon dioxide incubator (CO_2)	Kendro Laboratory, USA
ELISA plate reader	Tecan Sunrise, Salzburg, Austria
Field emission scanning electron microscope	JEOL Ltd, Japan
Flow cytometry FACS Canto II	Beckton Dickinson, USA
Hot plate	Thermo Scientific, USA
Ice maker	Scotsman Frimton, Italy
Light microscope	Olympus, Japan
Micropipette	Eppendorf, Germany
Microscope slide	Sailing Boat, China
Polysine slides	Thermo Scientific, USA
Rotary evaporator	Buchi, Switzerland
Shaker incubator	Interscience Sdn Bhd, Malaysia
Vortex	Scientific Industries, USA
Water bath	Memmert, Germany

3.4 Culture media

3.4.1 Nutrient broth

The nutrient broth prepared was mainly composed of 8 g nutrient broth powder, 5 ml glycerol, 5 g yeast extract, and 0.8 ml Tween 80. The mixture was dissolved in 1000 ml ddH_2O followed by sterilization by autoclave and stored at room temperature prior to use.

3.4.2 Nutrient agar

The nutrient agar prepared was mainly composed of 8 g nutrient powder being dissolved in 1000 ml ddH_2O. The medium was sterilized by autoclave and cooled down up to 50^oC before being dispensed aseptically into Petri dish plates. The plate agar medium was allowed to solidify prior to further storage at 4^oC.

3.4.3 Complete medium

The complete medium prepared was mainly composed of RPMI-1640 medium supplemented L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin solution. The mixture of the medium was completely resuspended.

3.5 Composition of solution

3.5.1 Carbol fuchsin solution (1%)

Carbol fuchsin staining solution was prepared by adding 100 ml of denatured alcohol to 50 g of phenol in a 1L conical flask. The mixture was mixed gently until completely dissolved. A hundred grams of basic fuchsin powder was added into the solution and well-stirred before adding in the remaining water to make up a total volume of 1000 ml. The solution was allowed to dissolve on a magnetic stirrer for a few hours and stored in a dark-coloured bottle.

3.5.2 Acid alcohol (3%)

The acid alcohol decolourizing solution was prepared by slowly adding 30 ml of concentrated hydrochloric acid into 970 ml of ethanol absolute. The solution was stored at room temperature.

3.5.3 Coomassie blue solution

Coomassie blue solution was prepared by dissolving 0.8 g of Coomassie blue in 100 ml of isopropanol and stirred completely. An addition of 260 ml ddH_2O was mixed into the solution and stored at room temperature. Ten milliliters of acetic acid were slowly added into 90 ml of Coomassie blue solution upon usage.

3.5.4 Ethanol solution (70%)

The ethanol solution prepared was mainly composed of 70 ml of 100% ethanol being dissolved in 30 ml ddH_2O. The solution was stored at room temperature.

3.5.5 Sodium hydroxide solution (3M)

The sodium hydroxide solution was mainly composed of 6 g of NaOH pellet being completely dissolved in 35 ml ddH_2O while stirring. The remaining volume of ddH_2O was added to the solution to make up a total volume of 50 ml.

3.5.6 Diluted hydrogen chloride (1M)

The diluted hydrogen chloride solution was mainly composed of 10 ml concentrated HCl (10M) being completely diluted in 90 ml ddH_2O by stirring.

3.6 Preparation of buffer

3.6.1 Phosphate buffer saline

Phosphate buffer saline (PBS) was prepared by diluting 450 ml of sterile ddH_2O with 50 ml of 1X PBS.

3.6.2 Fetal bovine serum (10%)

Fetal bovine serum was prepared by adding 100 ml of FBS into 1L of complete medium.

3.6.3 Trypan blue

Ten microliters of trypan blue were added into 10 µl of cell suspension and mixed well.

3.6.4 1X Rhodamine Phalloidin staining

A 0.1 g of bovine serum albumin (BSA) was added to 1 ml of PBS. One microliter of 1000X Rhodamine Phalloidin was in the working solution and mixed well. The freshly prepared Rhodamine-Phalloidin staining was stored on ice and kept out from light exposure.

3.6.5 Hoechst stain

One microliter of Hoechst stain was added into 199 µl of PBS and mixed well. The Hoechst stain was stored on ice and kept out from light exposure.

3.7 Liposomes

3.7.1 Culture of *M. smegmatis* mc^2155

M. smegmatis mc^2155 strain was grown in a medium comprised of 1% (w/v) yeast extract (Oxoid, UK), 0.5% (v/v) glycerol, 0.4% (v/v) Tween 80, in 8% nutrient broth at 37^{o}C with 200 rpm agitation for 48 hours.

3.7.2 Identification of *M. smegmatis* using Ziehl-Neelsen staining

Ziehl-Neelsen (ZN) stain, also known as Acid-fast staining, is mainly applied in the demonstration of acid-fast bacteria that belongs to the genus 'mycobacterium', which plays a crucial role as the infectious agent of tuberculosis. Due to the high lipid content of Mycobacteria cell walls that are extremely difficult to stain using ordinary method stains, ZN stain was used in this study to evaluate the purity of *M. smegmatis* culture. *M. smegmatis* was grown onto a nutrient agar plate within three days of incubation. A single colony was picked and fixed thinly smear onto a glass slide by heating. The smear was flooded with carbol fuschin and heated gently until fumes appeared before allowing it to stand for 5 minutes. Rinsed with water. Three percent of acid alcohol was poured over the smear for 2-5 minutes until light pink colour can be observed over the slide and rewashed. The smear was covered with methylene blue for 1-2 minutes and washed off. The back of the microscope slide was wiped cleanly and air-dried before proceeding with microscopic examination under a 100x oil immersion objective lens.

3.7.3 Lipid extraction

M. smegmatis was centrifuged at 3000 rpm for 30 minutes. Empty 50 ml corning tubes were weighed before the addition of culture media to determine the

weight of biomass. Pellet was resuspended in PBS and centrifuged at 3000 rpm for 10 minutes. A mixture of chloroform/methanol (2:1) was added to the tube and vortexed vigorously. The tube was sealed with parafilm and incubated in the shaker overnight at 37°C. The mixture was recentrifuged at 3000 rpm for 10 minutes. A hole was made in the materials formed at the interface before the lower phase was removed carefully. A hundred microliters of distilled water were added and gently mixed until the emulsion had disappeared. Removed the lower phase before adding a little spoon of sodium sulphate. The mixture was left incubated overnight at 4°C and re-centrifuged (3000 rpm for 10 minutes) on the next day to collect the supernatant.

The empty balloon was weighed before proceeding with mixture drying using a rotary evaporator. The balloon was reweighed once the sample has completely dried to determine the weight of lipids obtained. Extracted lipids were allowed to deposit in the Eppendorf tube before being further resuspended in 500µl of chloroform using a micropipette. Transferred into a sterile 25 ml balloon and dried with a rotary evaporator. A 500µl of sterile distilled water and sterile glass beads were then added and vortexed, alternated with incubation at 65°C until the total volume reached 9 ml. The sample was transferred into a 15 ml corning tube and distributed into three sterile glass bulbs. Stored the glass bulbs at -20°C before proceeding with the lyophilization process for 48 hours. The glass bulbs were kept for a long period of storage at 4°C.

3.7.4 Characterization of liposomes

Liposomes were characterized by Field Emission Scanning Electron Microscopy (FESEM) model FEI Quanta FEG 450 to examine the morphology of liposomes by dynamic light scattering using a Brookhaven ZetaPlus. Field emission scanning electron microscopy (FESEM) was performed at School of Health Sciences PPSK, Universiti Sains Malaysia (USM). The samples were initially diluted in ddH_2O

and loaded onto stub (Figure 3.1). The samples were dried up completely using desiccator before being mounted onto the FESEM sample stub (Figure 3.2). The gold coating was applied over the sample to inhibit charging upon being bombarded with an electron beam and refined on the secondary signal to produce good-quality images. Samples were further viewed under FESEM.

Figure 3.1 Stub for loading sample

Figure 3.2 Desiccator for drying sample

3.8 Study design

This was a cross-sectional study, which was conducted among TST-negative individuals, TST-positive individuals, and active pulmonary TB patients according to the Human Ethics Approval USM.

3.9 Study area

This study was conducted at Hospital Universiti Sains Malaysia (Hospital USM) Kubang Kerian and Immunology Research Laboratory, Universiti Sains Malaysia (USM), Kelantan, Malaysia.

3.10 Sample collection duration

All recruitment of participants was conducted from January 2021 until June 2022.

3.11 Study population

The cases of this study involved active pulmonary TB patients who were admitted to medical wards in Hospital USM. The TST-negative and TST-positive individuals were recruited among staff and students from USM. Documentation consent was obtained from all eligible participants after a detailed explanation of the study.

3.11.1 Active pulmonary TB patients

Inclusion criteria:

All participants diagnosed with pulmonary TB, sputum smear, and/or culture-positive, HIV-negative, without diabetes mellitus or other disease associated with

immunodeficiency and without immunosuppressive treatment, aged between 18 to 60 years old were enrolled in this study.

Exclusion criteria:

i. Age below 18 years old

ii. Use of immunosuppressive drugs

iii. HIV-Positive

iv. Pregnant women

v. Breastfeeding woman

vi. Use treatment for diabetes and hypertension

3.11.2 Tuberculin skin test

Inclusion criteria:

Healthy subjects who were included in this study served as a positive and negative control, based on Mantoux tuberculin skin test (TST) performance. An induration with 15 millimetres and above upon the skin test reaction is considered a positive reaction, meanwhile, an induration less than 5 millimetres is examined as a negative reaction. Individuals with positive reaction are classified as someone infected with TB bacteria meanwhile, individuals with negative reaction indicate to those who have never been infected with TB bacteria. They have been screened for eligibility and have none of the following exclusion criteria as stated below.

Exclusion criteria:

i. Age below 18 years old

ii. Presented with clinical symptoms and infection of TB

iii. Personal history of TB

iv. Unvaccinated with BCG

v. Use of immunosuppressive medication

vi. HIV-Positive

3.11.3 Sampling method

The selection of active pulmonary TB cases depended on the review of the medical history documented patient's folder. TST-negative and TST-positive individuals were selected through simple TST test sampling. All subjects who fulfilled the inclusion and exclusion criteria and were voluntarily interested in joining this study were recruited.

3.11.4 Sample size calculation

The sample size was determined according to the specific objectives 2, 3, and 4 by using F-tests ANOVA: Fixed effects, special, main effects, and interactions. The effect size of 0.4, α-error probability of 0.05, power = 1-β error probability of 0.8, numerator degree of freedom of 2, and the number of groups that equals 3 were applied. Hence, this yielded a total sample size of 66 participants divided equally between TST-negative individuals, TST-positive individuals, and active pulmonary TB patients (n=22 per group).

3.11.5 Written consent

All participants were informed and briefed about the project and written consent was obtained from each TST-negative individual, TST-positive individual,

and active pulmonary TB patient. Blood was collected after the written consent was documented.

3.12 Demographic data and sample isolation

3.12.1 Recruitment and data collection

All participants were informed and briefed about the project and written consent was obtained from TST-negative individuals, TST-positive individuals, and active pulmonary TB patients. The demographic data of each participant such as full name, registration number, address, date of birth, gender, ethnicity, age, sex, race, contact number, onset, and family history of TB were obtained through the interview of each individual and the medical record of patients.

3.12.2 Collection of blood

Twelve ml of peripheral blood was withdrawn from the peripheral vein of each TST-negative individual, TST-positive individual, and active pulmonary TB. The blood was collected in a BD Vacutainer® Ethylenediaminetetraacetic acid (EDTA) tube and stored at room temperature during transportation. Samples were processed in the Tissue Culture Laboratory of the Immunology Department in Hospital USM, within three hours of blood collection for immunophenotyping of DCs subsets by flow cytometry.

3.12.3 Collection of plasma

Collected blood in the EDTA tube was centrifuged at 700 x g for 10 minutes. The resulting plasma was then transferred into a 1.5 ml microtube and stored at -80^{o}C for future studies.

3.12.4 Isolation of peripheral blood mononuclear cells

PBMCs were isolated from peripheral blood according to the Lymphoprep™ separation procedure. Plasma and blood pellets were separated and collected for different purposes. The remaining blood in the EDTA tube was reconstituted with Phosphate-Buffer Solution with a ratio of 1:1 and mixed by inversion. Diluted blood was carefully overlaid onto the wall of 15ml Falcon tubes containing Lymphoprep™ solution by using a Pasteur pipette. The tubes were titled sideways during the addition of reconstituted blood without breaking the surface plane and centrifuged at 500 x g room temperature for 30 minutes with brake-off, to isolate the buffy coat PBMCs.

The mononuclear cell layer was gently aspirated by swirling rotation, transferred, and pooled into a new 15 ml Falcon tube containing 2 ml PBS using a Pasteur pipette. The remaining PBS was topped into the tube up to 15 ml, gently mixed, and centrifuged at 500 x g room temperature for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended with PBS and centrifuged at 500 x g for 7 minutes as the second wash. The supernatant was discarded, and the cell pellet was resuspended with 1 ml PBS. The cell suspension was kept on ice while performing cell counting using Hemacytometer.

Ten µl of the cell suspension was added into a new 1.5 ml microtube and mixed with 10 µl of trypan blue exclusion dye using a 1:1 dilution factor. Ten ul of the mixture was loaded onto the hemacytometer and viewed under a light microscope. Viable and dead cells were counted using the cell counter, and the cell counts were calculated using the formula below:

$$\text{Cell viability (\%)} = \frac{\text{Average live cells}}{\text{Average total cells}} \text{ x } 100$$

$$\text{Cell density } \left(\frac{\text{cells}}{\text{ml}}\right) = \frac{\text{Average live cells x dilution factor}}{\text{Volume of a square (ml)}}$$

$$\text{Cell number (cells)} = \text{Cell density } \left(\frac{\text{cells}}{\text{ml}}\right) \text{ x volume (ml)}$$

3.12.5 Cell seeding with liposomes incubation

The cell suspension of PBMCs from TST-negative individuals, TST-positive individuals, and active pulmonary TB patients were cultured into 96 well plates, with a concentration of 1 x 10^6 cells/ml. Two hundred microliters of fresh complete medium RPMI supplemented with 10% fetal bovine serum (FBS) were added into each well of TST-negative and TST-positive individuals, meanwhile, only a 100 µl of fresh complete medium RPMI were added into the well of active pulmonary TB patients. A hundred microliters of cell suspension were added and resuspended into each well. Liposomes (50 µg/ml) were exposed to the PMBCs, meanwhile, positive controls were stimulated with LPS (100 ng/ml) derived from *Escherichia coli*. As for the negative controls, it only comprised complete medium. The 96 well plates were incubated and maintained at 37ºC with a 5% CO_2 incubator for 24 hours and harvested after stimulation for further analyses.

3.13 Flow cytometry

3.13.1 Immunophenotyping assessment on dendritic cells

Upon overnight exposure with liposomes, the cells were harvested by spinning down at 500 x g for 10 minutes. A staining buffer containing 10% FBS and PBS was prepared. The supernatant was removed, and the cells were resuspended and washed twice with a staining buffer. Antibodies cocktail was prepared, added to the cells, and stained on ice in the dark for 30 minutes to identify mDCs using the following antibodies: CD11c-APC; HLA-DR-APC/Cy7; CD123-PE, CD86-PE/Cy7; dead cell

exclusion-7-AAD (Table 3.4). Cells were centrifuged to wash off the staining buffer and the supernatant was removed. Stained cells were resuspended with PBS prior to flow cytometry acquisition (Figure 3.3).

Table 3.4 Optimized concentration and function of conjugated antibodies for PBMCs staining

DCs Fluorochrome-labelled antibody	Optimized concentration	Function
APC-CD11c	1:400	Marker for mDCs
APC/Cy7-HLA-DR	1:400	Marker for DCs
PE-CD123	1:20	Surface marker for plasmacytoid DCs
PE/Cy7-CD86	1:20	Surface marker for activation of DCs
7-AAD-Dead cell exclusion	1:800	To acquire live cells and increase the accuracy of the result

3.13.2 Gating strategy of flow analysis

This study focuses on identifying myeloid dendritic cells, upon exposure to liposomes stimuli, which requires an appropriate gating strategy.

Total leukocytes were first identified by low forward scatter (FSC) and low side scatters (SSC) gating and viable cells population were further acquired. The subsequent gating identifies 2 different populations of DCs; mDCs and pDCs, followed by the identification of specific DCs populations (HLA-DR^+CD11c^+, HLA-DR^+CD11c^-, HLA-DR^-CD11c^+ and HLA-DR^-CD11c^-). Activation marker of DCs, $CD86^+$ was then obtained (Figure 3.4).

Figure 3.3 Flow cytometry Canto II

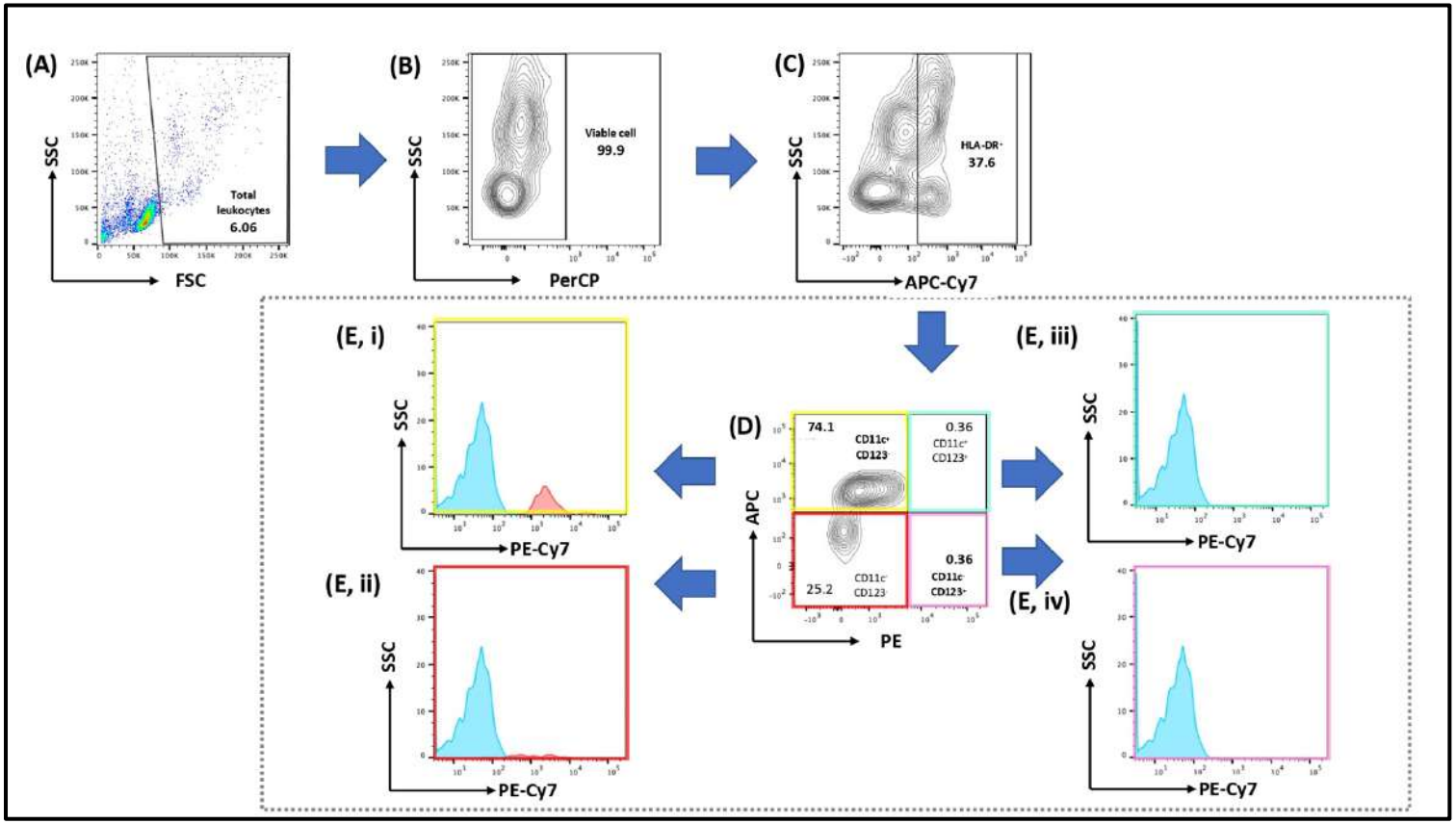

Figure 3.4 Gating strategy for identification of dendritic cells

Representative FACS dot plots showing (**A**) total leukocytes identified from forward and side scatter and further gated into (**B**) viable cell population. (**C**) Upon the exclusion of dead cells, the HLA-DR$^+$DCs population was gated. (**D**) mDCs and pDCs subsets were acquired accordingly followed by (**E**) CD86$^+$DCs population. (**E, i**) represented CD86$^+$ from mDCs, (**E, ii**) showed CD86$^+$ obtained from double negative populations, (**E, iii**) represented CD86$^+$ obtained from double positive populations, and (**E, iv**) showed CD86$^+$ obtained from pDCs. The percentage of each cell population was shown in bold numbers indicating DCs gating of HLA-DR$^+$CD11c$^+$ and CD86$^+$ cells, respectively.

3.14 Liposomes-Msmeg uptake analysis by dendritic cells

3.14.1 Confocal microscopy analysis

Confocal analysis was carried out to investigate the uptake and internalization of liposomes-Msmeg inside the DCs. Cell mortality was also monitored to verify the biocompatibility of liposome-Msmeg. The PBMCs cells were cultured with FITC-conjugated liposomes overnight. On day 2, the cells were harvested by PBS washing. A hundred microliters of 1X Rhodamine-Phalloidin staining were added to the cell suspension and the 96-well plate was incubated overnight. On day 3, the cells were harvested and undergo 3 short washing steps. Two hundred microliters of Hoechst 33342 stain were added to the cells and incubated in the dark for 15 minutes at room temperature. The cells were washed and resuspended with hundred microliters of PBS. Fifty microliters of cell suspension were smeared onto a poly-L-lysine-coated glass slide and air-dried. The slide was mounted with an immunofluorescence mounting medium and covered with a coverslip before being viewed under a Nikon A1r Confocal microscope, operated by NIS Elements Viewer software (Figure 3.5). Data were analysed using FIJI software.

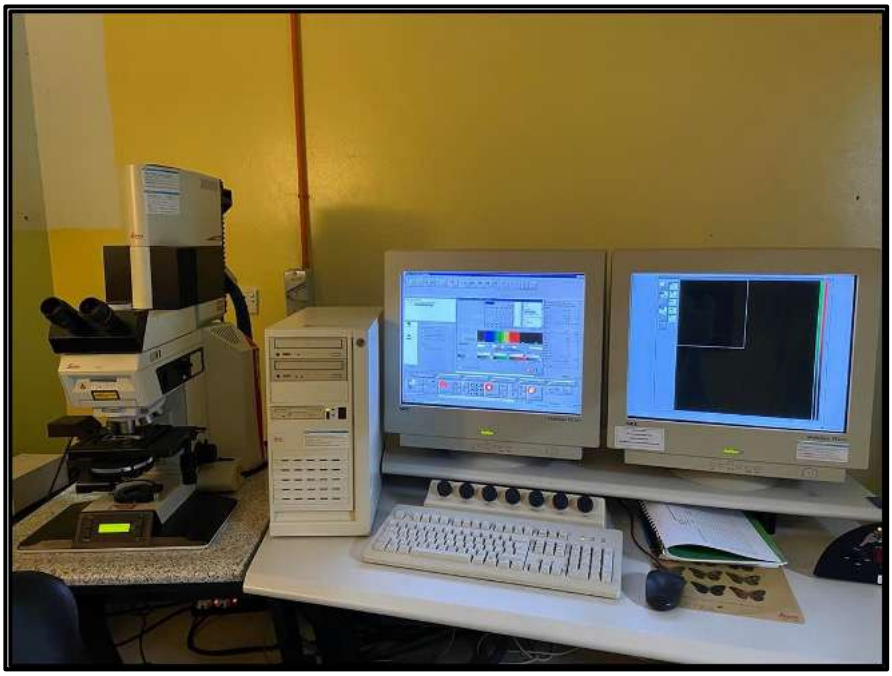

Figure 3.5 Confocal microscopy to view the samples

3.14.2 Field emission scanning electron microscopy

Field emission scanning electron microscopy analysis was performed to examine the uptake and effect of liposomes-Msmeg within DCs. Overnight cultured PBMCs cells were harvested and resuspended with ddH_2O. The cell samples were initially fixed with primary fixation of McDowell Trump fixative at 4^oC for 24 hours. Three washing steps with ddH_2O have been carried out for 10 minutes. Secondary fixation was further performed with cold 1% osmium tetroxide at room temperature for 1-2 hours. The cells were washed with ddH_2O twice for 10 minutes and undergoes 15 minutes of dehydration with different graded of acetone (35%, 50%, 75%, 95%, and 100%), respectively. Subsequently, the cells were soaked in a 1:1 Hexamethyldisilane (HMDS)/Acetone for 15 minutes and continued for another 15 minutes in a 100% HMDS solution. The cells were dried completely overnight and diluted with ddH_2O and coated with a thick layer (20.9 nm) of gold to cover the conductivity imbalance (Figure 3.6). Gold has been an optimal and the most

frequently used sputter coating material for high-resolution imaging due to its high conductivity and relatively small fine size. The liposomes were mounted onto a metal stab and air-dried to minimize specimen distortion and observed under the microscope subsequently. Finally, the samples were ready to be viewed under FESEM microscopy (Figure 3.7).

Figure 3.6 Sputter coating to coat samples

Figure 3.7 Field emission scanning electron microscopy to view the samples

3.15 Cytokines profile analysis

Freshly isolated immature DCs (10^6 cells/ml in 24-well plates) were incubated overnight with liposomes-Msmeg. PBS-incubated cells served as a negative control. The culture supernatants were collected and stored at -80^{o}C until assayed. The concentrations levels of IFN-γ, IL-12p70, and IL-4 were determined from culture supernatants by ELISA with commercially available assay kits according to standard procedures. Briefly, the 96-well plates were coated with diluted Capture Antibody and coated overnight at 4^{o}C. The washing steps were performed and repeated 4 times each, except for the last wash which requires 5 times washing with a soaking step for 30 seconds to 1 minute per wash. All incubations were carried out at room temperature with shaking on a plate shaker for 500 rpm. The plates were blocked with 1X Assay Diluent A for 1 hour. In the meantime, six two-fold serial dilutions of the standard concentrations for each cytokine were prepared accordingly. The prepared standards and sample were then added into the respective well and incubated for 2 hours. The washing step was performed before the addition of diluted Detection Antibody solution and 1 hour incubation period. After the next washing step, diluted Avidin-HRP solution was added, incubated for 30 minutes, and washed, ultimately. A freshly mixed TMB Substrate solution was added in each well and incubated for 20 minutes in the dark. The addition of Stop Solution concludes the final step of sample processing via ELISA and the plate was read at the 450 nm absorbance within 15 minutes. The standards and blank wells regulate as positive and negative controls, respectively.

3.16 Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software. The data were analysed for normality and log-transformed by one sample T-test to compare between two groups, and two-way ANOVAs with Tukey's multiple comparison test to correlate between multiple groups.

CHAPTER 4

RESULTS

This chapter elaborates on the findings of this study. The findings include a demographic characteristic that comprises the clinical characteristics and predisposing factors in TST-negative individuals, TST-positive individuals, and active pulmonary TB patients. The mean percentages of mDCs and pDCs subsets, the expression markers of DCs, and immunoassays cytokines: IL-12p70, IFN-γ, and IL-4 have been further examined.

4.1 Sociodemographic factors of TST-negative individuals, TST-positive individuals, and active pulmonary tuberculosis patients

Data were analysed using standard statistical methods, GraphPad Prism analysis. The sociodemographic criteria are shown in Table 4.1. A total of 22 TST-negative individuals, 20 TST-positive individuals, and 19 active pulmonary TB patients were recruited in this study from January 2021 to June 2022. In the TST-negative group, 36.33% were males and 63.64% were females. Within the TST-positive groups, 35% of males and 65% of females have been enrolled, meanwhile, all the active pulmonary TB patients recruited were males. Furthermore, the enrolled active pulmonary TB patients were mostly older adults with a percentage of 84.21%. In contrast, the highest percentage of participants in both TST-negative and TST-positive individuals were middle-aged adults, with 63.64% and 60%, respectively. None of the young adults in both active pulmonary TB patients and TST-positive individuals have been enrolled in this study. On the other hand, the participation of middle-aged adults of the active pulmonary TB patients was 10.52%, whereas the percentage of young adults in TST-negative individuals who participated was

27.27%. Both the older adults in TST-negative and TST-positive individuals demonstrated moderate participation with 9.09% and 40%, respectively. In addition, most of the active pulmonary TB patients enrolled in this study consisted of 89.47% Malays, 5.26% Chinese, and 5.26% of the other race. Meanwhile, more than 95% of the Malays in both TST-negative and TST-positive individuals, with less than 5% of the Chinese have been recruited.

Table 4.1 Demographic data of recruited groups

	TST-negative, n (%)	**TST-positive, n (%)**	**Active pulmonary TB, n (%)**	***p* value**
Age group				0.835
18-30 (young adults)	6 (27.27)	0	1 (5.26)	
31-44 (middle-aged adults)	14 (63.64)	12 (60)	2 (10.52)	
Above 45 (older adults)	2 (9.09)	8 (40)	16 (84.21)	
Gender				0.108
Male	8 (36.36)	7 (35)	19 (100)	
Female	14 (63.64)	13 (65)	0	
Race				**0.002**
Malay	21 (95.45)	19 (95)	17 (89.47)	
Chinese	1 (4.55)	1 (5)	1 (5.26)	
Others	0	0	1 (5.26)	

p value from two-way ANOVA test

4.2 Clinical data on tuberculosis and its predisposing factor

The predisposing factors of TB infection are shown in Table 4.2. History of tuberculosis and completion of treatment were crucial among active pulmonary TB patients. Both active pulmonary TB patients and TST-positive individuals showed the highest percentage of the absence of TB history, with 78.95% and 80%, respectively. A similar pattern was observed in the household contact and non-household contact of TB infection for both groups. The result showed that around 5.26% had TB household contact, meanwhile, an estimation of 15.79% obtained TB non-household contact. Besides, more than 78% of the active pulmonary TB patients and 95% of the TST-positive individuals have not received any treatment for TB. In the meantime, 21.05% of the active pulmonary had completed the treatment for TB infection. For the TST-positive individuals, 10% had completed the treatment while 5% were still under ongoing treatment. Furthermore, in active pulmonary TB patients, more than 50% of them were reported as active smokers, followed by 26.32% of the non-smokers, and 15.79% of the passive smokers. Meanwhile, TST-positive individuals showed a higher percentage of non-smokers with 90% and only 10% as active smokers.

Table 4.2 Predisposing factors of tuberculosis infection

	TST-negative, n (%)	**TST-positive, n (%)**	**Active pulmonary TB, n (%)**	***p* value**
History of TB				**0.002**
Within household contact	0	1 (5)	1 (5.26)	
Non-household contact	0	3 (15)	3 (15.79)	
No TB contact	0	16 (80)	15 (78.95)	
Treatment status				0.478
Completed treatment	0	2 (10)	4 (21.05)	
No treatment (X-ray cleared)	0	19 (95)	15 (78.95)	
Ongoing treatment	0	1 (5)	0	
Smoking status				**0.048**
Active smoker	2 (9.10)	2 (10)	11 (57.89)	
Passive smoker	0	0	3 (15.79)	
Non-smoker	20 (90.9)	18 (90)	5 (26.32)	

p value from two-way ANOVA test

The pre-examination criterion in association with active pulmonary TB patients has been demonstrated in Table 4.3. This group showed that 73.68% had been diagnosed with positive smear and positive culture while 26.32% were reported as a positive smear and negative culture. In addition, 21.05% of the participants were diagnosed as negative smear and positive culture, with 5.26% having negative smear and negative culture, respectively. On the other hand, all the active pulmonary TB patients were not under the prescription of immunosuppressive drugs. Other factors such as HIV-positive, pregnancy, breastfeeding, and the prescription of diabetic and hypertension drugs have been excluded in both groups.

Table 4.3 Pre-examination criterion of smear in active pulmonary TB

	Active pulmonary TB, n (%)	***p* value**
AFB smear with culture		**0.0001**
Positive smear, positive culture	13 (73.68)	
Positive smear, negative culture	1 (26.32)	
Negative smear, positive culture	4 (21.05)	
Negative smear, negative culture	1 (5.26)	

p value from one sample t-test

4.3 Liposomes production and characterization methods

This study used liposomes to evaluate DCs activation upon exposure to liposomes. The liposomes derived from *M. smegmatis* were produced at the School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan. Ziehl-Neelsen staining was performed to identify and confirm the purity of *M. smegmatis* culture (Figure 4.1). The development of liposomes was completed through the dehydration-rehydration method as described in chapter 3. The characterization of liposomes has been performed via FESEM to measure their size range (Figure 4.2).

4.3.1 Ziehl-Neelsen staining

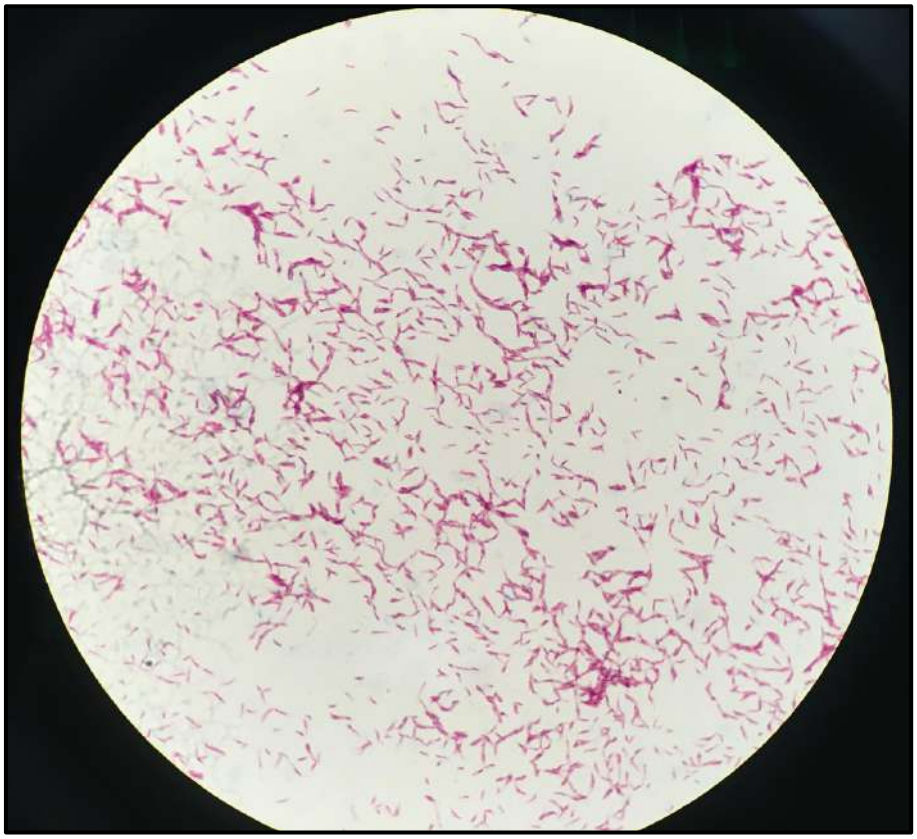

Figure 4.1 Microscopic image of *M. smegmatis* culture by Ziehl-Neelsen staining under 100x objective lens with immersion oil

The microscopic image represents the pinkish-red slender rods of *M. smegmatis* staining with a light blue background upon culturing Ziehl-Neelsen staining. This observation was obtained through the retaining of carbol fuchsin staining and decolorization with acid-alcohol by the large amounts of mycolic acid in the bacteria cell wall. Hence, supporting that *M. smegmatis* is an acid-fast bacteria species. *M. smegmatis* was an ideal choice to replace the BCG for *in-vitro* testing due to its fast growth rate and non-pathogenic characteristics. A small portion of the liposomes, 50 ug, was further characterized by FESEM.

4.3.2 Field emission scanning electron microscope

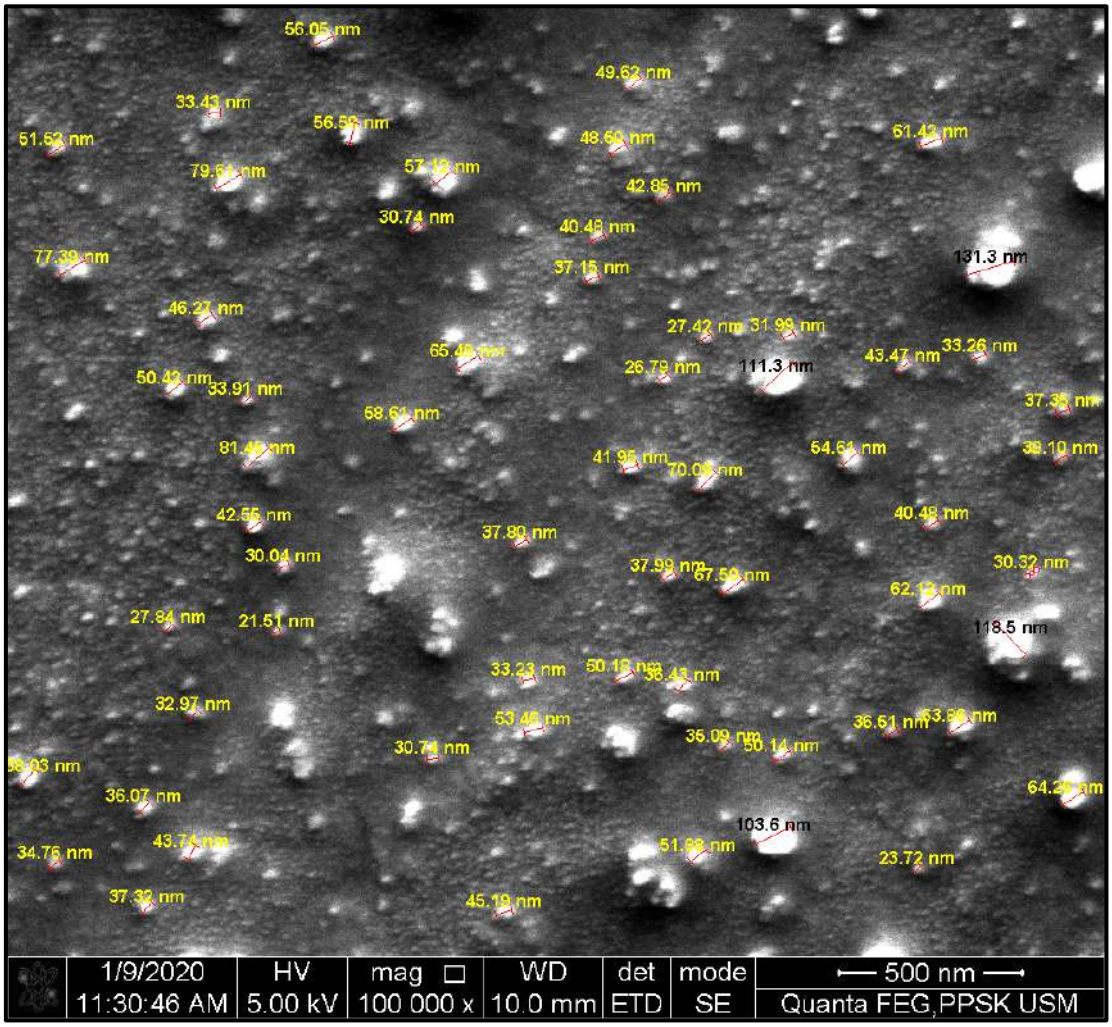

Figure 4.2 Field emission scanning electron microscopy of *M. smegmatis* liposomes

The characterization of liposomes via FESEM analysis demonstrated bright and spherical structure formation as shown in Figure 4.2. The size of the liposomes is varied, with a size ranging from 20-135 nm with most of them being less than 100 nm. These observations highly referred to the classification of small unilamellar vesicles (SUVs) liposomes. This type of liposomes is bounded by single lipid bilayer and has been preferable due to its good uptake by cells. The liposomes were initially coated with gold sputter coating and the image was captured under 100000x magnification.

4.4 Surface marker expression of dendritic cells between TST-negative individuals, TST-positive individuals, and active pulmonary tuberculosis patients upon exposure to liposomes by flow cytometry

The surface marker expression of DCs, HLA-DR-APC/Cy7 and CD11c-APC, and immune activation of the surface molecule, CD86-PE/Cy7 upon exposure to liposomes were executed by cell surface staining and FACS analysis. Samples were acquired with FACS Canto II, interpreted using Flowjo®, and analysed with GraphPad Prism version 9.0. Table 4.5 represents the percentage of DCs subsets; myeloid and plasmacytoid DCs of the study cohort.

4.4.1 Percentage of dendritic cells and their subsets, myeloid dendritic cells and plasmacytoid dendritic cells

Table 4.4 Profile of myeloid and plasmacytoid dendritic cells of TST-negative individuals, TST-positive individuals, and active pulmonary TB patients upon exposure to *M. smegmatis* liposomes

Dendritic cells	**TST-negative (n=22)**			**TST-positive (n=20)**			**Active pulmonary TB (=19)**			***p* value**
	Negative control	**Positive control**	**Liposomes**	**Negative control**	**Positive control**	**Liposomes**	**Negative control**	**Positive control**	**Liposomes**	
HLA-DR$^+$ (%)	30.76 ± 10.94	28.88 ± 9.28	34.24 ± 13.41	31.36 ± 11.47	31.65 ± 12.57	34.68 ± 13.27	13.80 ± 9.97	15.39 ± 10.51	24.27 ± 13.39	**0.0001**
Myeloid dendritic cells										
CD11c$^+$ CD123$^-$ mDCs (%)	59.10 ± 14.87	56.52 ± 14.13	60.77 ± 19.42	58.98 ± 20.55	55.27 ± 19.08	57.87 ± 21.66	61.70 ± 21.72	63.72 ± 19.43	53.02 ± 25.78	0.839
CD86$^+$ (MFI)	2334.71 ± 646.95	1595.58 ± 417.05	2042.79 ± 569.58	2295.75 ± 626.06	1642.12 ± 447.93	1998.43 ± 589.48	1566.25 ± 447.21	1304.84± 466.78	1472.84 ± 392.98	**0.0001**
Plasmacytoid dendritic cells										
CD11c$^-$ CD123$^+$pDCs (%)	0.610 ± 0.480	0.908 ± 0.696	0.192 ± 0.255	0.758 ± 0.517	1.43 ± 0.847	0.381 ± 0.606	0.346 ± 0.944	0.333 ± 0.398	0.719 ± 1.759	**0.037**

The data extracted in Table 4.4 shows the percentage of each DCs subsets in the study cohorts. The assessment of HLA-DR$^+$DCs is presented in Figure 4.3, followed by CD11c$^+$CD123$^-$mDCs in Figure 4.4, and CD11c$^-$CD123$^+$pDCs in Figure 4.5. Meanwhile, Figure 4.6 shows the MFI of CD86$^+$mDCs. *p<0.05, ***p<0.0001

4.4.2 Assessment of HLA-DR class II molecule on dendritic cells

The dot plot in **Figure 4.3** shows the expression of antigen-presenting cells, HLA-DR$^+$, upon PBMCs exposure to different stimuli in TST-negative, TST-positive, and active pulmonary TB groups. The DCs of active pulmonary TB patients upon being exposed to liposomes show lower significant stimulation of HLA-DR$^+$ than the DCs of TST-negative and TST-positive groups which were exposed to liposomes. There are no significant differences between the liposomes group with the negative and positive control groups in active pulmonary TB group. On the other hand, the DCs of TST-positive individuals which was exposed to liposomes demonstrated no significant difference in the expression of HLA-DR$^+$ when compared to both negative and positive control groups. Likewise, a similar trend is observed in the DCs of TST-negative group which was exposed to liposomes in comparison to the negative and positive control groups.

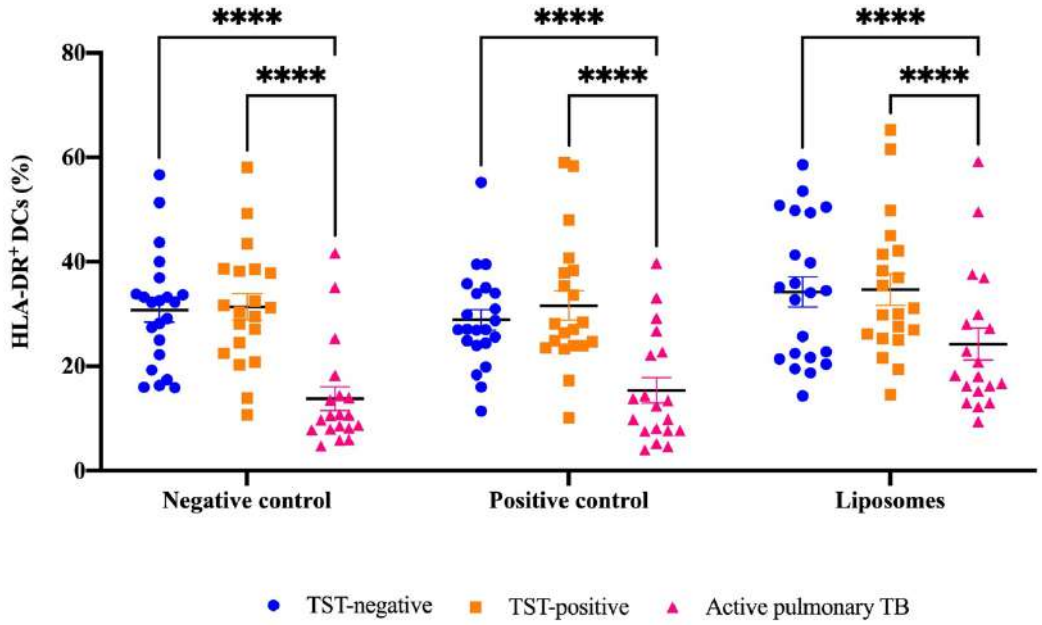

Figure 4.3 Percentage of surface marker HLA-DR$^+$ in different groups and conditions. The result is presented as mean ± SEM of percentage of HLA-DR$^+$. ***p<0.0001

4.4.3 Assessment on CD11c and CD123 DCs expressions of HLA-DR^{+}

4.4.3(a) HLA-DR^{high}CD11c^{+}CD123^{-}mDCs

Figure 4.4 shows the expression of the specific DCs population, CD11c^{+}CD123^{-}mDCs, on PBMCs obtained from the study cohort. There is no significant difference between all the groups proposed. However, active pulmonary TB group cultured with liposomes exhibited a lower expression of CD11c^{+}CD123^{-} mDCs than TST-negative and TST-positive groups which were cultured with liposomes. Likewise, the expression of CD11c^{+}CD123^{-}mDCs upon exposure to liposomes in the active pulmonary TB group is lower when compared to the negative and positive control groups. As for the exposure of liposomes in the TST-positive group, their CD11c^{+}CD123^{-}mDCs level is higher than the positive control group but similar to the negative control. Meanwhile, the expression of CD11c^{+}CD123^{-}mDCs upon being exposed to liposomes in TST-negative group is increased when compared to the negative and positive controls.

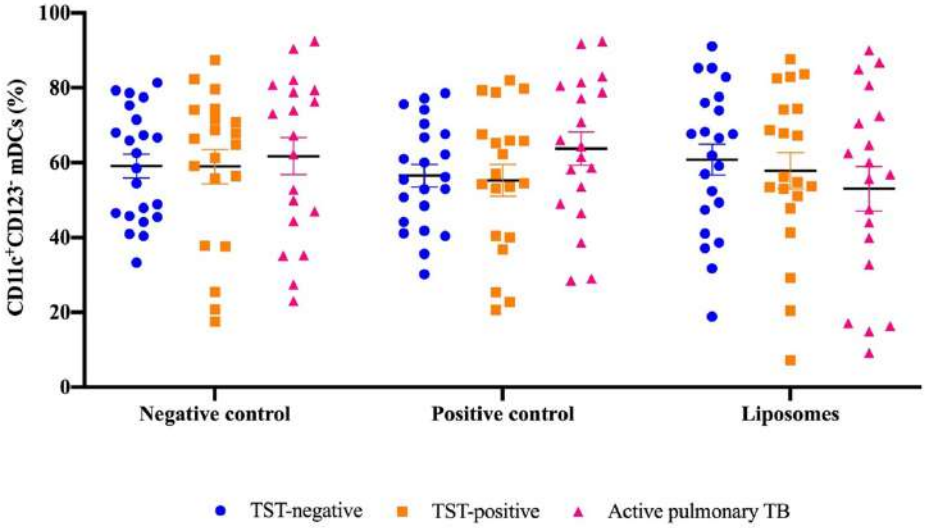

Figure 4.4 Percentage of surface markers CD11c^{+}CD123^{-}mDCs in different groups. The result is presented as mean ± SEM of percentage of CD11c^{+}CD123^{-} mDCs. No statistically significant difference in surface marker CD11c^{+}CD123^{-}mDCs expression was observed between all groups.

4.4.3(b) HLA-DR^{low}CD11c^{-}CD123^{+}pDCs

Figure 4.5 represents the expression of the specific DCs population, CD11c^{-}CD123^{+}pDCs, on PBMCs obtained from the study cohort. There is no significant difference between all the groups proposed. The exposure of liposomes in active pulmonary TB group shows an increased expression of CD11c^{-}CD123^{+}pDCs compared to the liposomes of TST-negative and TST-positive groups. Similarly, the expression trend of liposomes in the active pulmonary TB group is higher than in the negative and positive groups. For the liposomes of the TST-positive group, CD11c^{-}CD123^{+}pDCs stimulate the lowest expression in comparison to the negative and positive controls. A similar result can be observed in the liposomes of the TST-negative group, in which the expression of CD11c^{-}CD123^{+}pDCs is diminished when compared to the negative and positive control groups.

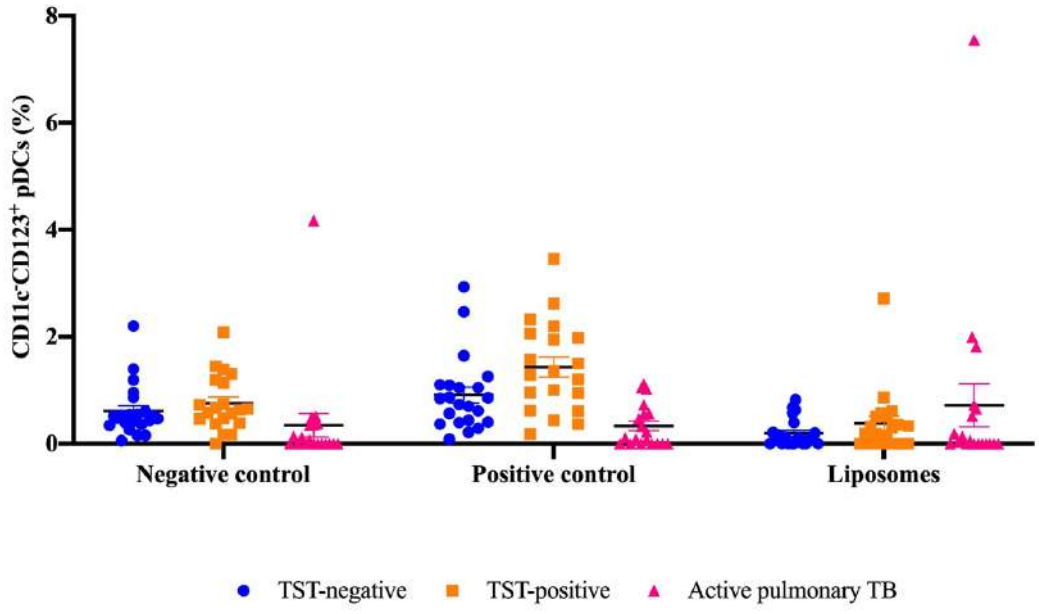

Figure 4.5 Percentage of surface markers CD11c^{-}CD123^{+}pDCs in different groups. The result is presented as mean ± SEM of percentage of CD11c^{-}CD123^{+}pDCs. No statistically significant difference in surface marker CD11c^{-}CD123^{+}pDCs expression was observed between all groups.

4.4.4 Assessment on CD86^{+} expression of CD11c^{+}CD123^{-}mDCs

The dot plot in Figure 4.6 shows the mean fluorescence intensity (MFI) of CD86^{+}mDCs on PBMCs compared to the study cohort. There is no significant difference between the expression of CD86^{+}mDCs in the liposomes of active pulmonary TB with the liposomes of TST-negative and TST-positive groups. However, the liposomes of the active pulmonary TB group observe a significant increase**,** when compared with the positive control group. The comparison between the liposomes of active pulmonary TB and the negative control groups shows that the expression of CD86^{+}mDCs is lower in the liposomes of the active pulmonary TB group but is not significant. In addition, the negative control of the active pulmonary TB group demonstrated a decrease significant when compared to the positive control of the active pulmonary TB group. As for the liposomes in the TST-positive group, an increase significant is only observed in comparison to the positive control group. There is no significant difference between the liposomes and negative control in the TST-positive group. The negative control of the active pulmonary TB group demonstrated a significant decrease as opposed to the positive control of the active pulmonary TB group. In the liposomes of the TST-negative group, there is a significant increase when compared to the positive control group but is not significant to the negative control group. Besides, the negative control the of TST-negative group shows a higher significant difference than the positive control group.

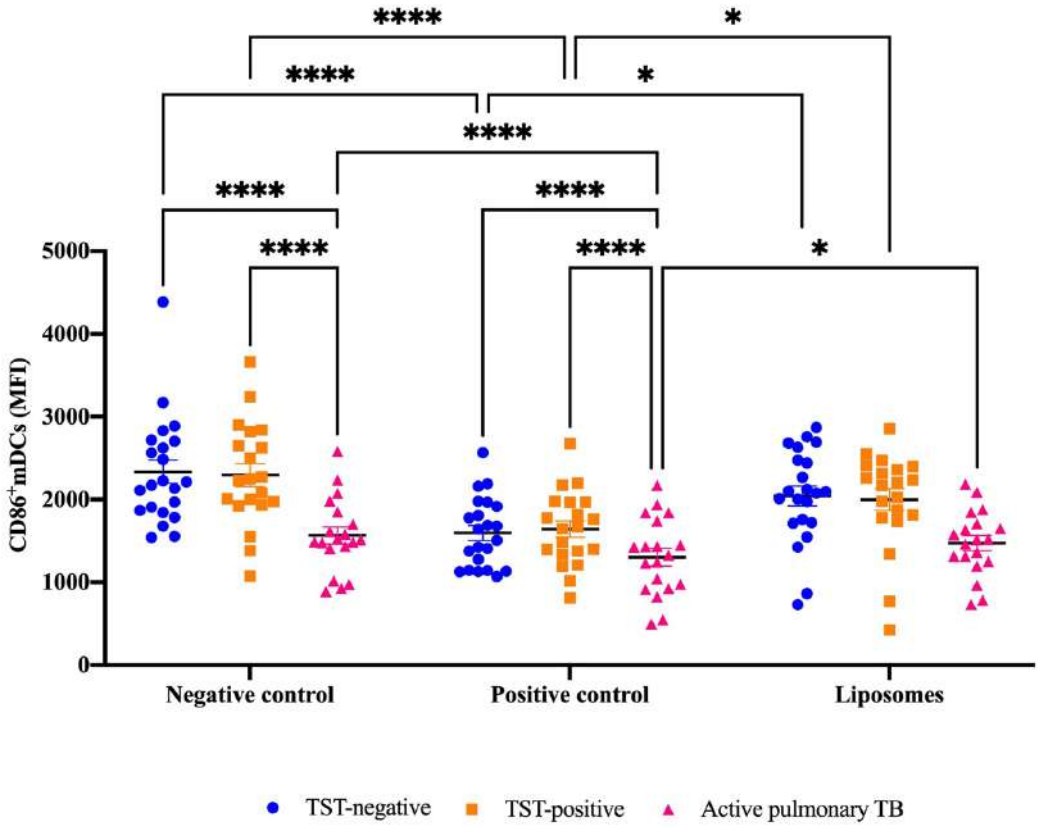

Figure 4.6 MFI of $CD86^{+}$mDCs in different groups. The result is presented as mean ± SEM of MFI of $CD86^{+}$mDCs. *p<0.05, ***p<0.0001

4.5 Cytokine secretion

Figure 4.7 represent the expression of cytokine secretion in the presence of liposomes among TST-negative individuals, TST-positive individuals, and active pulmonary TB patients. The DCs of active pulmonary TB which was exposed to liposomes group secrete a significant increase of IL-4 concentration in comparison to the DCs of TST-positive group exposed to liposomes. There is no significant difference between the DCs of active pulmonary TB and TST-negative groups exposed to liposomes. However, the DCs in TST-positive group exposed to liposomes demonstrate diminished IL-4 production when compared to the DCs in TST-negative group cultured with liposomes. In addition, the DCs of active pulmonary TB exposed to liposomes group exhibit highly significant concentrations of IL-12p70 upon being compared to the TST-positive and TST-negative groups, respectively. It can be observed that the DCs of active pulmonary TB cultured with liposomes group show a significant increase in IFN-γ levels when compared to the liposomes of the TST-positive group. The comparison of IFN-γ production between the DCs of TST-positive and TST-negative groups exposed to liposomes exhibits a significant difference.

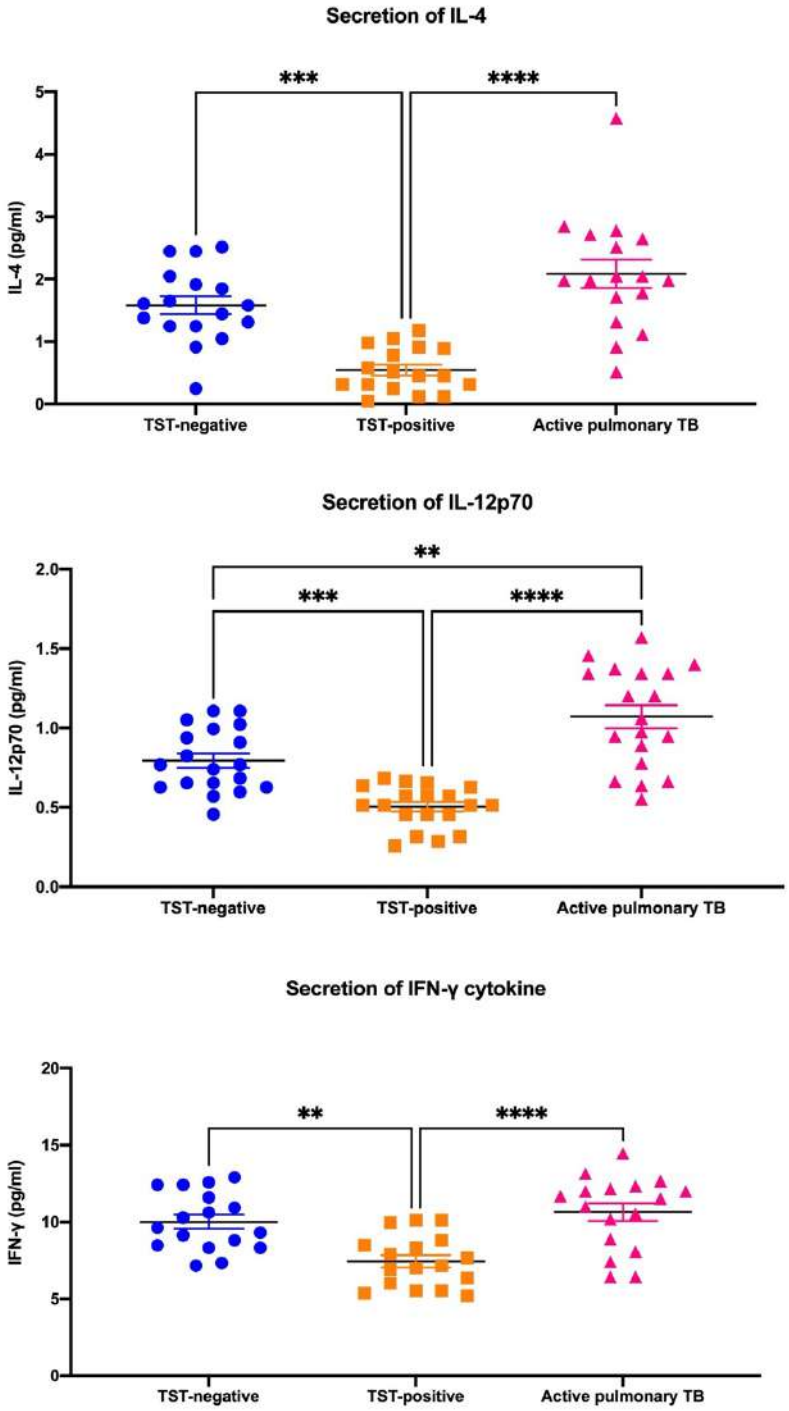

Figure 4.7 Secretion of cytokines, IL-4, IL-12p70, and IFN-γ. The result is presented as mean ± SEM of $CD86^{+}$mDCs. $^{**}p<0.001$, $^{***}p<0.0001$

4.6 Association study

4.6.1 Association between $CD11c^{+}CD123^{-}$mDCs and cytokine secretion

The trend of each cytokine with $CD11c^{+}CD123^{-}$mDCs (Figure 4.8) shows that there is no association between cell surface marker and cytokine secretion in all the study cohort.

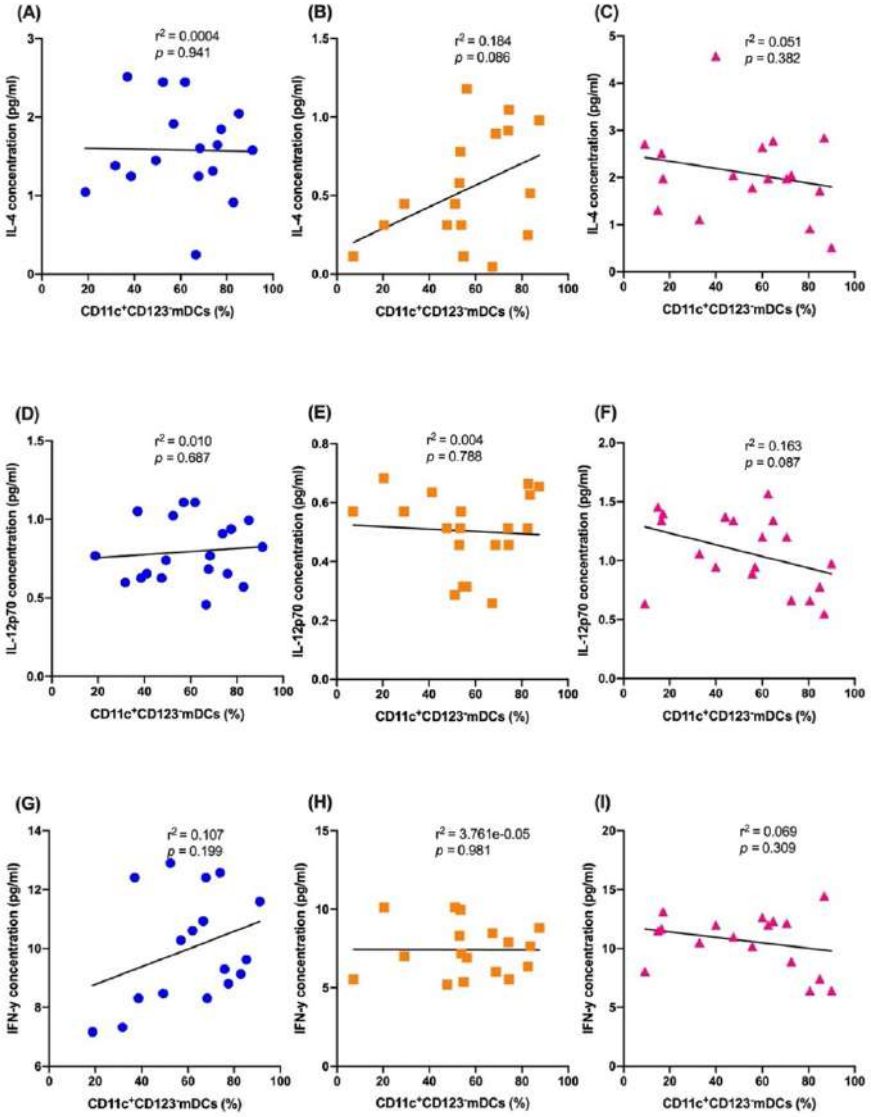

Figure 4.8 Association between secretion of each cytokine with $CD11c^{+}CD123^{-}$mDCs in TST-negative individuals (blue dots plots), TST-positive individuals (orange dot plots), and active pulmonary TB patients (pink dot plots). The result is presented as mean ± SEM of the percentage of $CD11c^{+}CD123^{-}$mDCs. No statistically significant difference in the secretion of each cytokine with $CD11c^{+}CD123^{-}$mDCs between all groups.

4.6.2 Association between $CD86^+$mDCs and cytokine secretion

In contrast, Figure 4.9 shows that IL-4 cytokine and $CD86^+$mDCs in TST-negative individuals are significantly associated. However, there are no associations between IL-4 cytokine and $CD86^+$mDCs in both TST-positive and active pulmonary TB groups.

On the other hand, the association between IL-12p70 level and $CD86^+$mDCs is only observed in TST-negative and active pulmonary TB groups. In TST-positive group, there is no association between the concentration level of IL-12p70 and $CD86^+$mDCs.

Meanwhile, the association between IFN-γ and $CD86^+$mDCs is only presented in TST-negative group. As for the TST-positive and active pulmonary groups, there are no associations observed between IFN-γ cytokine and $CD86^+$mDCs.

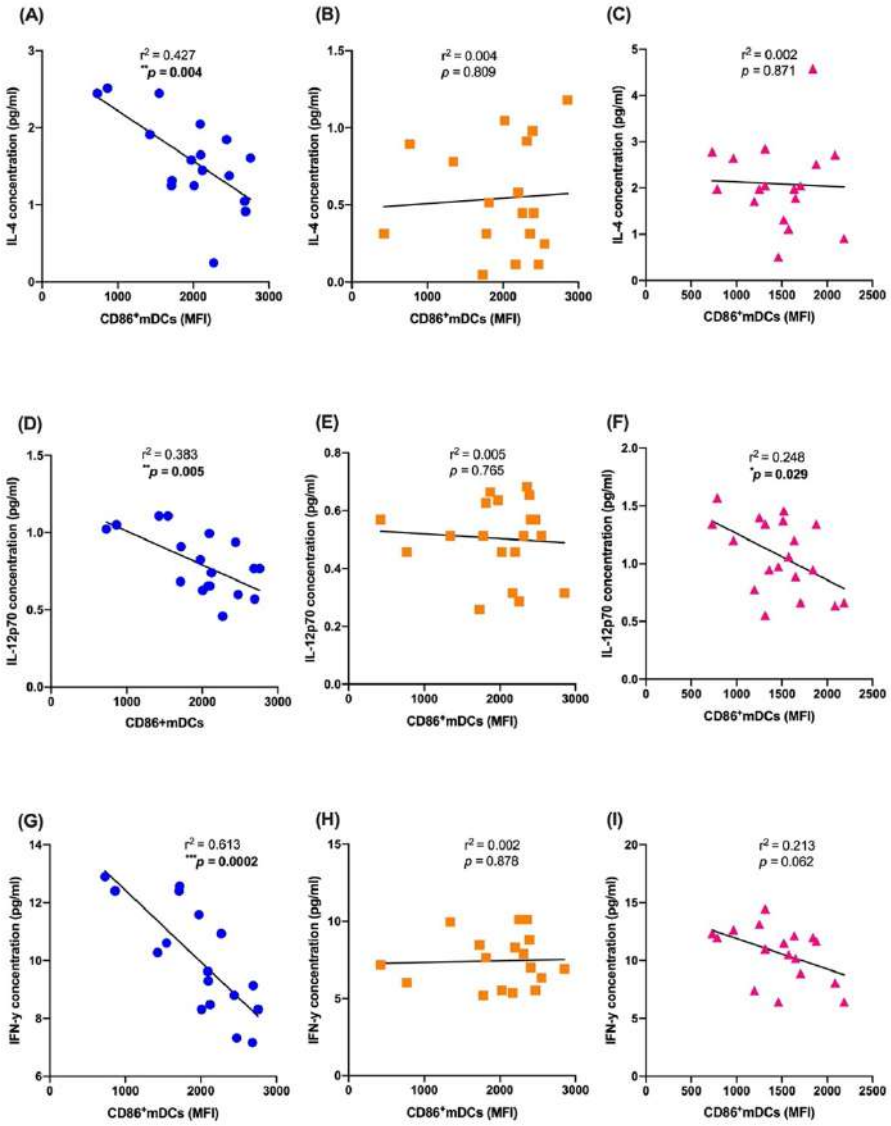

Figure 4.9 Association between secretion of each cytokine with $CD86^{+}$mDCs in TST-negative individuals (blue dot plots), TST-positive individuals (orange dot plots), and active pulmonary TB patients (pink dot plots). The result is presented as mean ± SEM of the MFI $CD86^{+}$mDCs. *p<0.05

4.7 Uptake and encapsulation of liposomes by dendritic cells

The interaction between liposomes and DCs was mainly examined based on the uptake of liposomes by DCs, the expression of surface markers, and the levels of cytokine expression. The study on the uptake of liposomes by DCs was illustrated by FESEM and confocal microscopy which resolve detailed and sharp images on the uptake of liposomes within DCs.

4.7.1 Field emission scanning electron microscopy

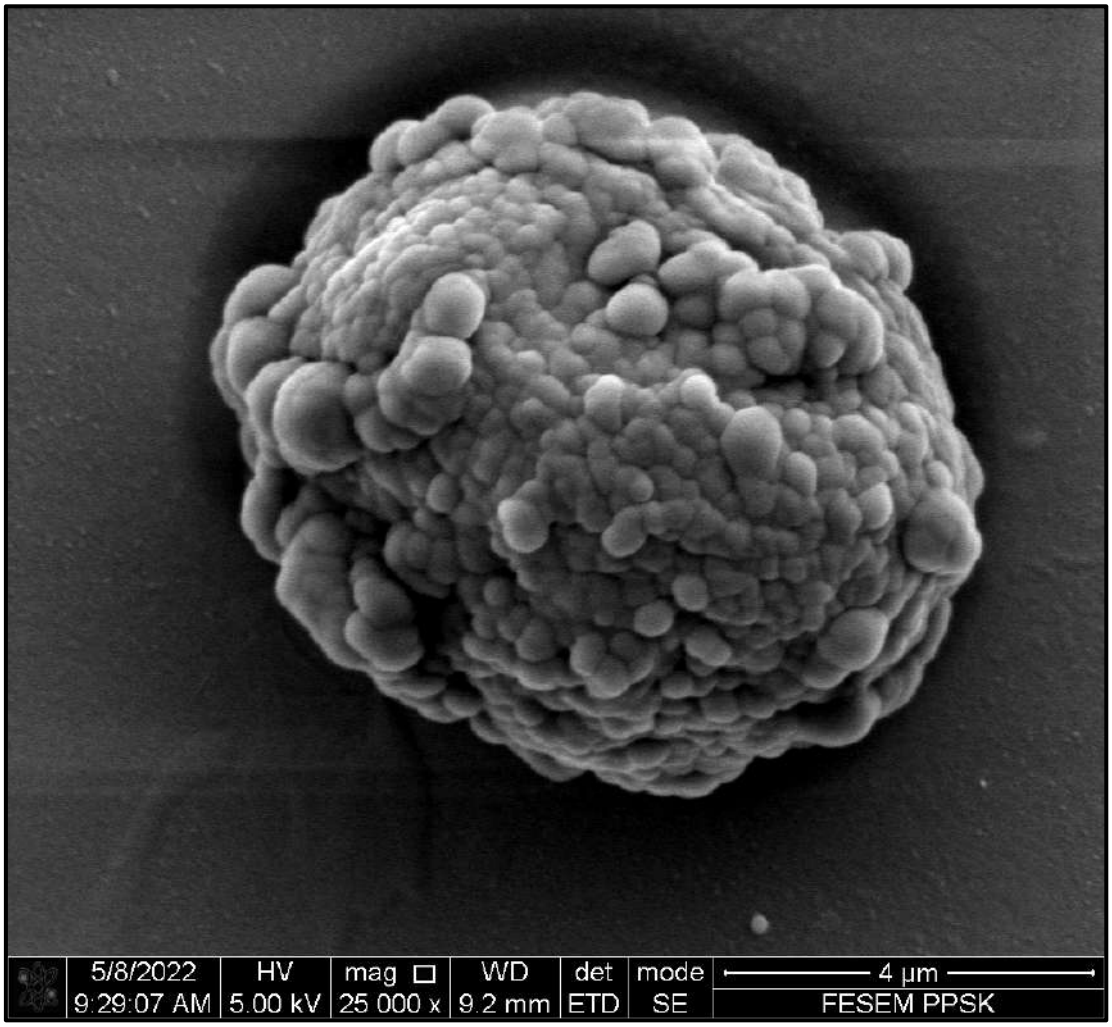

Figure 4.10 Morphology of DCs in TST-negative individuals without stimulators as assessed by field emission scanning electron microscope

The DCs image of TST-negative individual showed the circular shape of the cells (Figure 4.10). Cells were cultured with complete medium overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 25000x magnification.

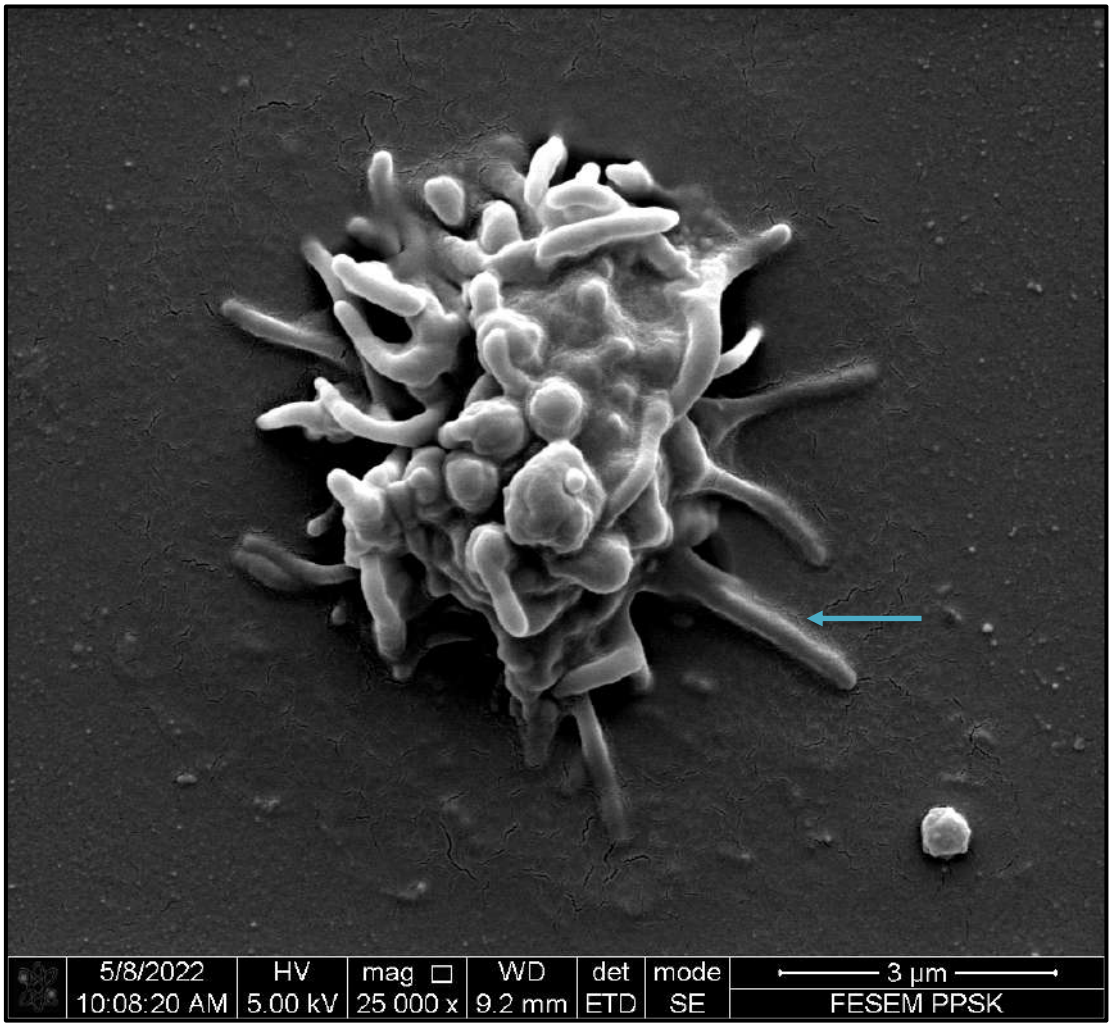

Figure 4.11 Morphology of DCs in TST-negative individuals stimulated with LPS as assessed by field emission scanning electron microscope

The DCs image of TST-negative individual shows a high number and extended dendrites as shown by the blue arrow (Figure 4.11). Cells were cultured with LPS stimulant in complete medium overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 25000x magnification.

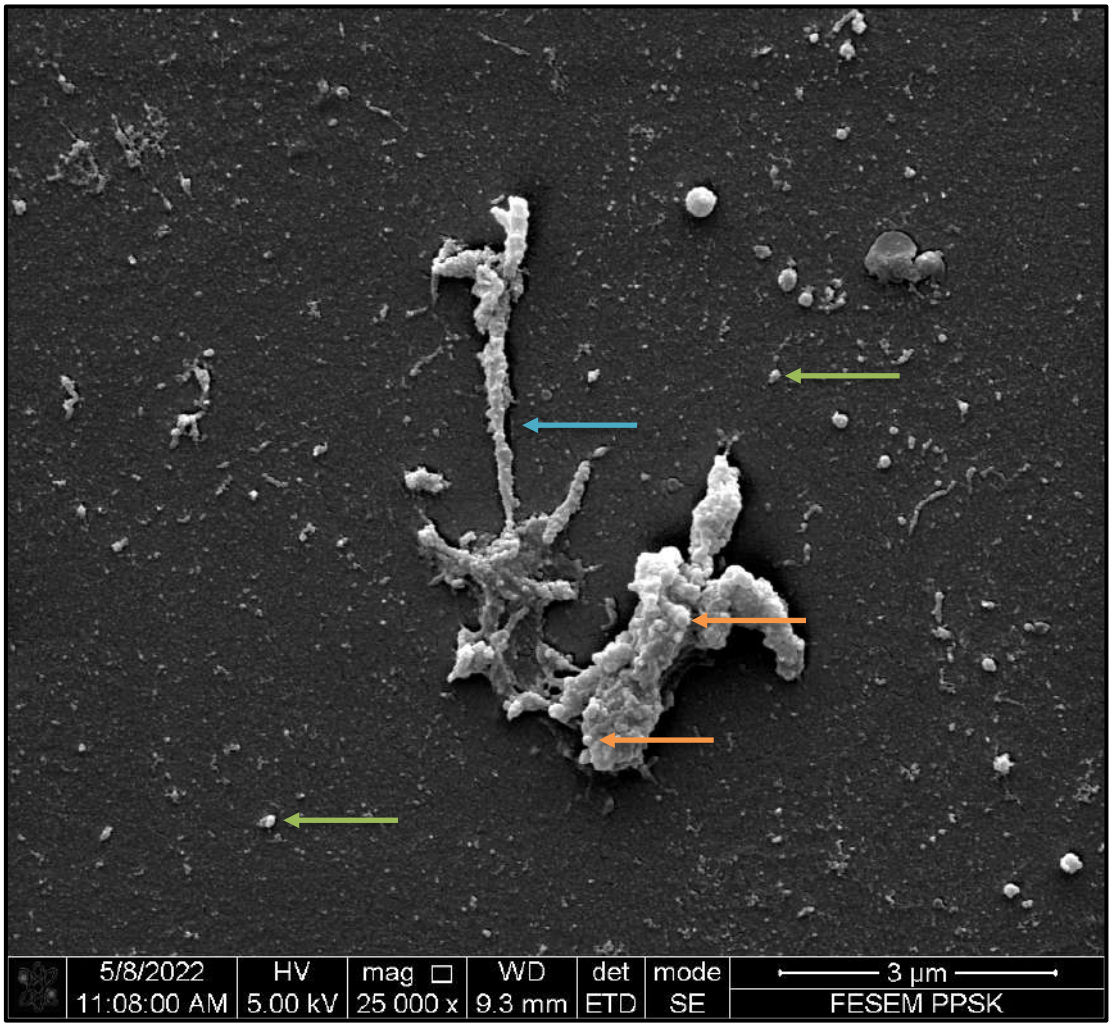

Figure 4.12 Morphology of DCs in TST-negative individuals stimulated with liposomes as assessed by field emission scanning electron microscope

The DCs image in TST-negative individual shows long dendritic protrusions of two combined DCs as pointed by the blue arrow (Figure 4.12). The engulfment of liposomes by DCs is observed based on the big circular formation on the surface of DCs as shown by the orange arrow. Other liposomes which are not uptake by the DCs mostly scattered next to the mature DCs, as pointed by the green arrows. Cells were cultured with liposomes in complete medium overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 25000x magnification.

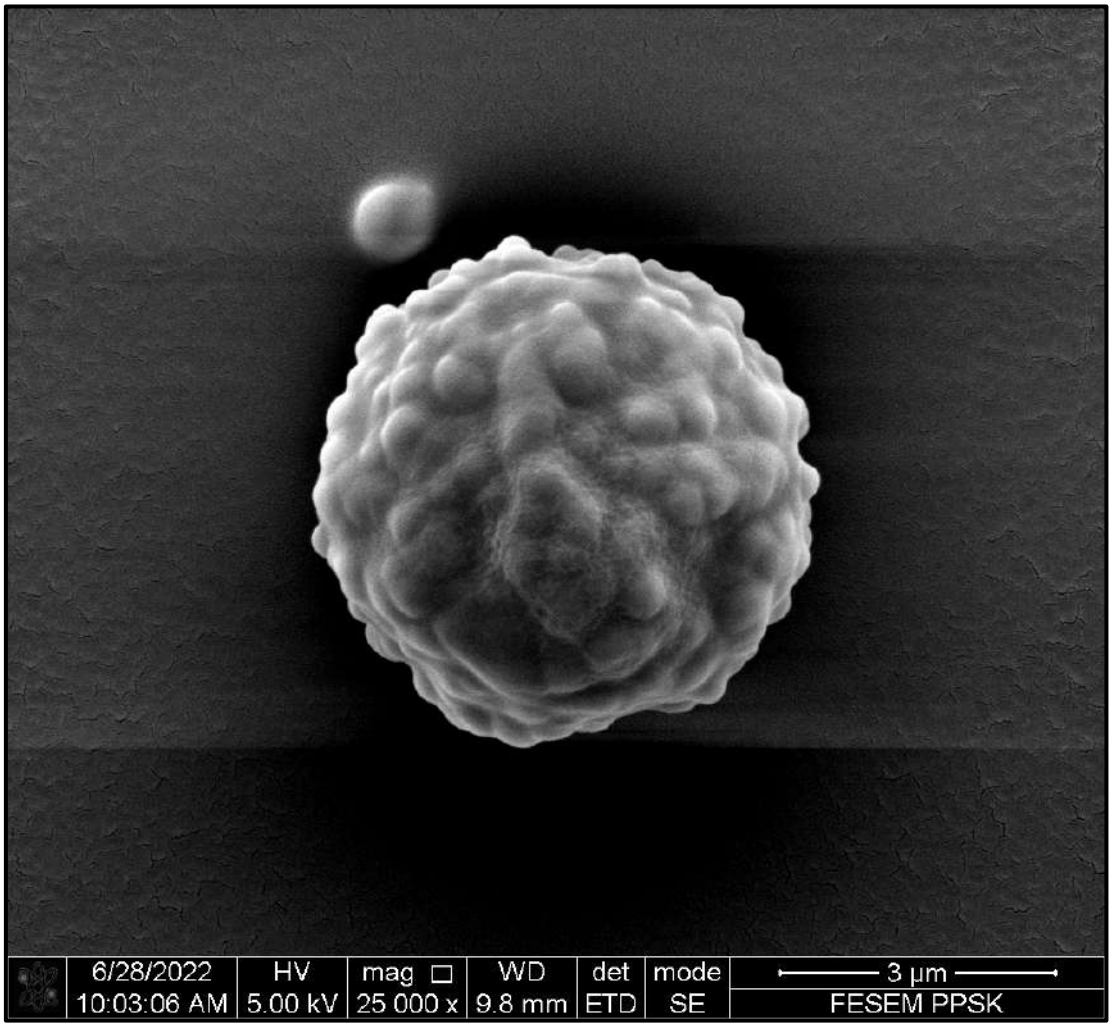

Figure 4.13 Morphology of DCs in TST-positive individuals without stimulators as assessed by field emission scanning electron microscope

The DCs image in TST-positive individual shows the circular shape of cells with the formation of unclear lumps on the surface of cells (Figure 4.13). Cells were cultured with complete medium overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 25000x magnification.

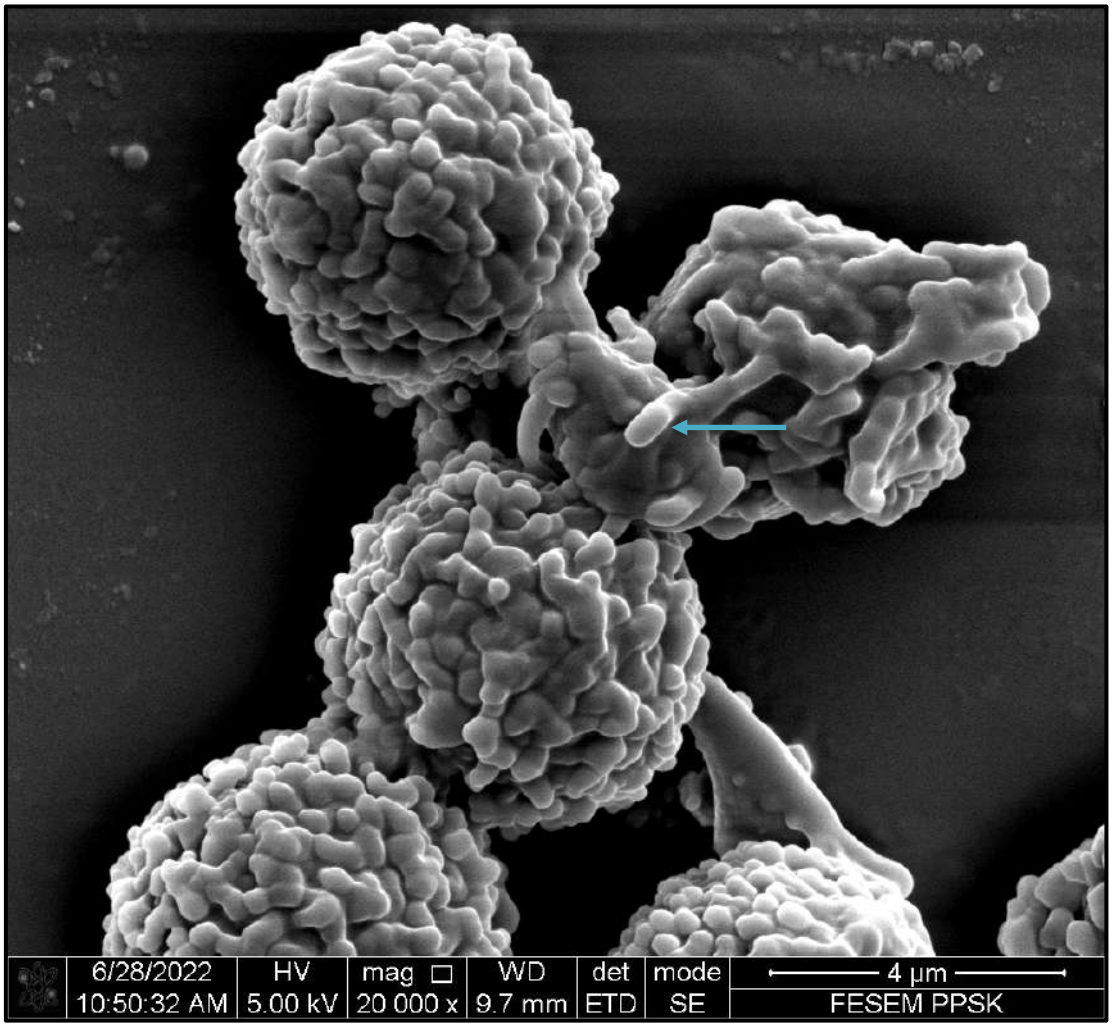

Figure 4.14 Morphology of DCs in TST-positive individuals stimulated with LPS as assessed by field emission scanning electron microscope

The DCs image of TST-positive individual shows the cells are bonded to one another with the formation of short extended dendrites as shown by the blue arrow (Figure 4.14). Cells were cultured with LPS stimulant overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 20000x magnification.

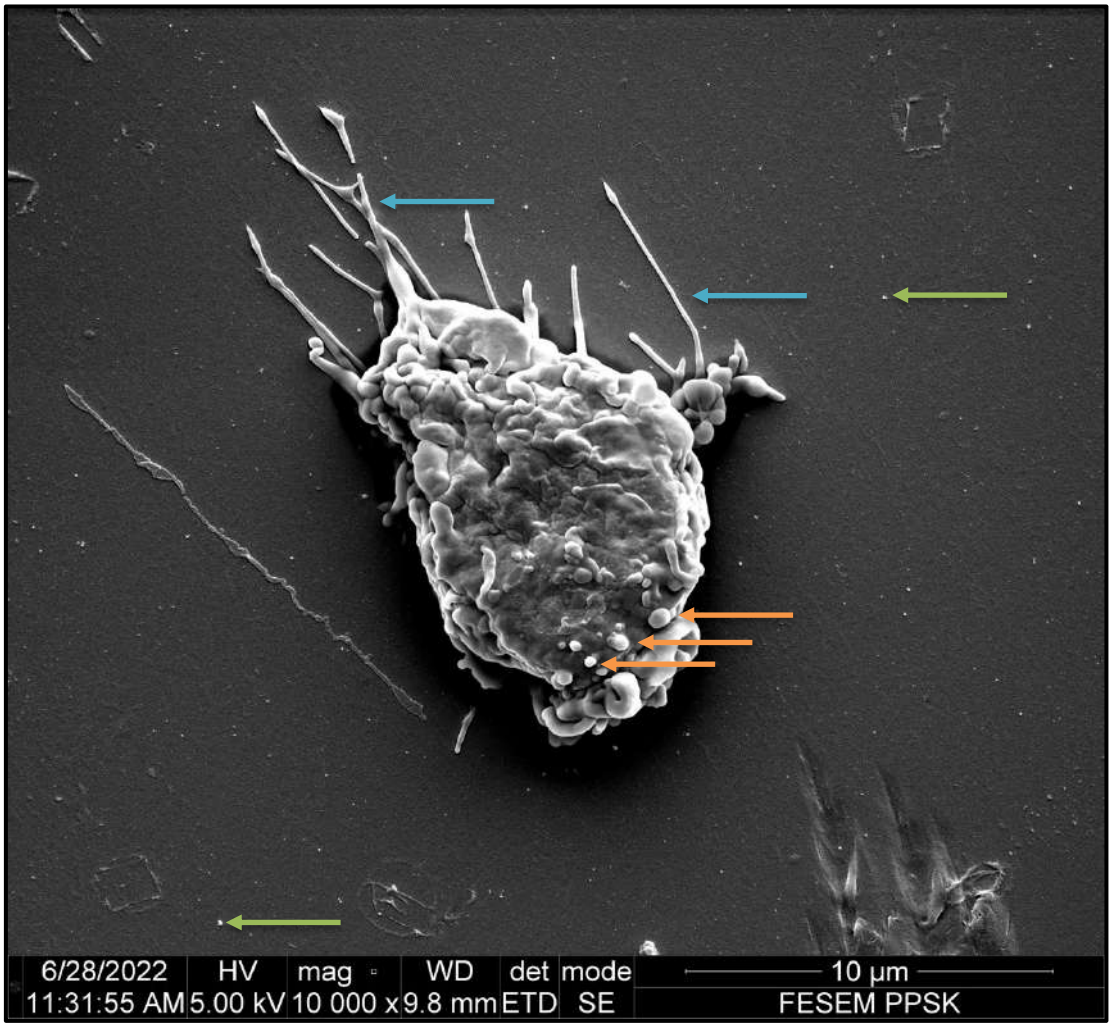

Figure 4.15 Morphology of DCs in TST-positive individuals stimulated with liposomes as assessed by field emission scanning electron microscope

The DCs image of TST-positive individual shows long dendritic protrusions of cells as pointed by the blue arrows (Figure 4.15). The engulfment of liposomes by DCs is observed based on the large circular formation on the surface of DCs as shown by the orange arrows. Other liposomes which are not uptake by the DCs mostly dispersed surrounding the cells as pointed by the green arrows. Cells were cultured with liposomes overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 10000x magnification.

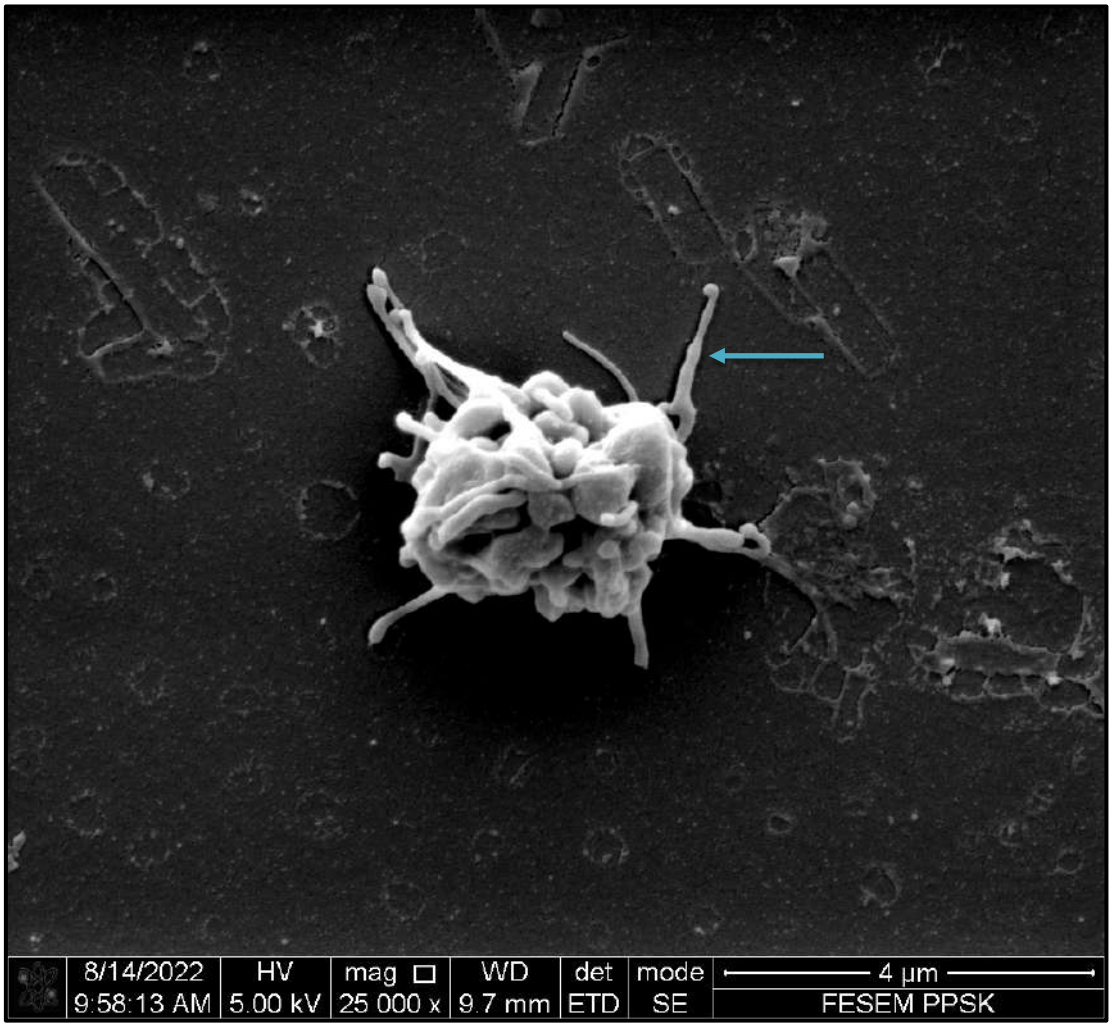

Figure 4.16 Morphology of DCs in active pulmonary TB patients without stimulators as assessed by field emission scanning electron microscope

The DCs image of active pulmonary TB patients shows the circular shape of the cells with the presence of short dendrites as pointed by the blue arrow (Figure 4.16). Cells were cultured with complete medium overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 25000x magnification.

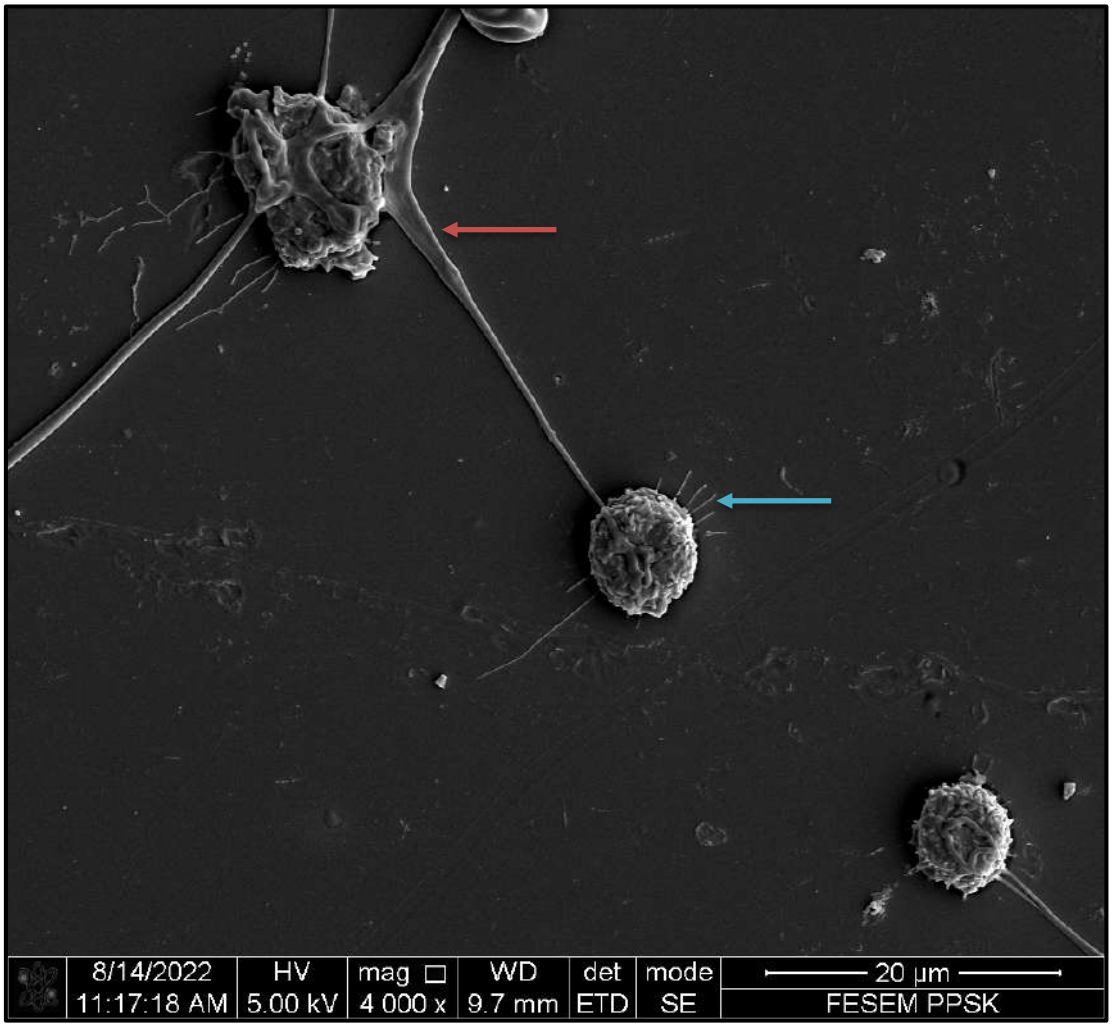

Figure 4.17 Morphology of DCs in active pulmonary TB patients stimulated with LPS as assessed by field emission scanning electron microscope

The DCs of active pulmonary TB shows the circular shapes of cells that are jointed with another similar cell by the formation of long dendritic protrusions as pointed by the red arrow (Figure 4.17). In addition, extended dendrites in various directions also can be observed as shown by the blue arrow. Cells were cultured with LPS stimulant overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 4000x magnification.

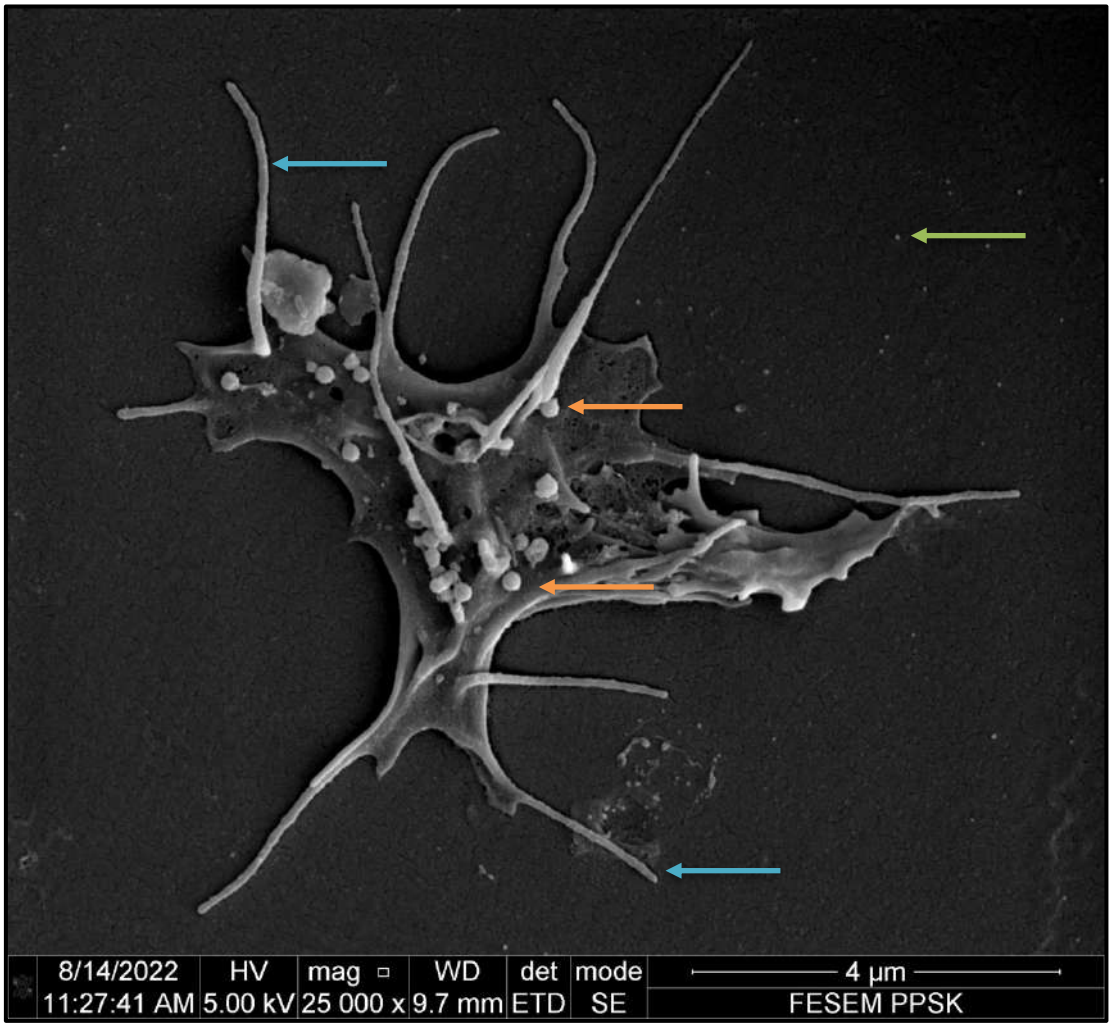

Figure 4.18 Morphology of DCs in active pulmonary TB patients stimulated with liposomes as assessed by field emission scanning electron microscope

The DCs image of the active pulmonary TB group shows long dendritic protrusions cells as pointed by the blue arrows (Figure 4.18). The engulfment of liposomes by DCs is observed based on the large circular formation on the surface of DCs as shown by the orange arrows. Other liposomes which are not uptake by the DCs mostly scattered surrounding the cells as pointed by the green arrow. Cells were cultured with liposomes overnight, seeded on a polylysine-coated coverslip, prepared, and viewed under 25000x magnification.

4.7.2 Confocal microscopy

The current study intends to investigate the internalization of liposomes inside DCs on active pulmonary TB compared to TST-negative and TST-positive individuals. The DCs were stained with liposomes conjugated-FITC 488 and actin filament-stained Rhodamine Phalloidin 543, followed by the visualization under confocal microscopy. The images captured the presence of liposomes and DCs, respectively. The merging images showed the encapsulation of liposomes by DCs as shown in Figures 4.19, 4.20, and 4.21.

The merged image in Figure 4.19 illustrates the obvious uptake of liposomes by DCs of TST-negative individuals, as pointed out by the orange arrows. The same spots are observed in both liposomes-conjugated FITC and actin-conjugated Rhodamine. On the contrary, the uptake of liposomes in the DCs of TST-positive individuals demonstrates a rather dim signal with fewer spots as shown by the orange arrow in Figure 4.20. The signal of the liposomes-conjugated FITC and actin-conjugated Rhodamine is much weaker in the TST-positive group compared to the TST-negative group. Meanwhile, Figure 4.21 exhibited a strong uptake of liposomes by the DCs of active pulmonary TB patients via the bright signal observed as shown by the orange pointer.

Liposomes stained with FITC-488 nm | Dendritic cells stained with Rhodamine-Phalloidin-543 nm | Merge image

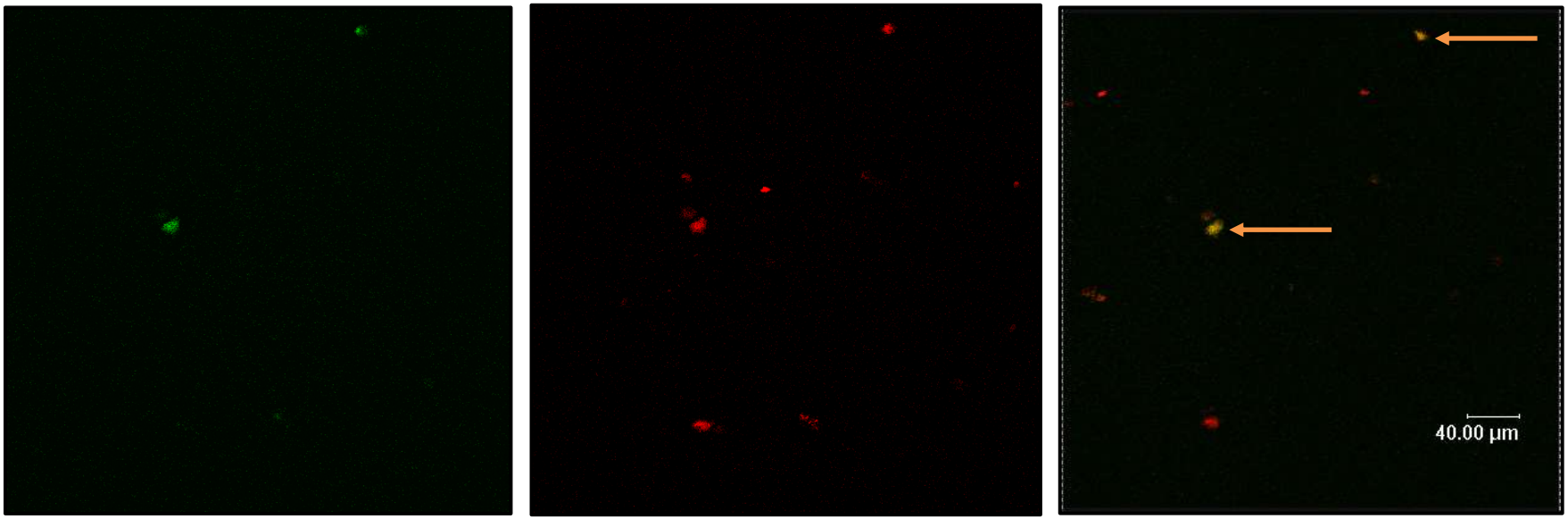

Figure 4.19 Confocal microscopy analysis of the interaction between DCs and liposomes in TST-negative individual.

Two immunofluorescence colours; in which the red colour represents DCs, meanwhile, the green colour illustrates the liposomes. Both images were overlaid to observe the uptake of liposomes by DCs as pointed by the orange arrows. Scale bars: 40 µm

Liposomes stained with FITC-488 nm | Dendritic cells stained with Rhodamine-Phalloidin-543 nm | Merge image

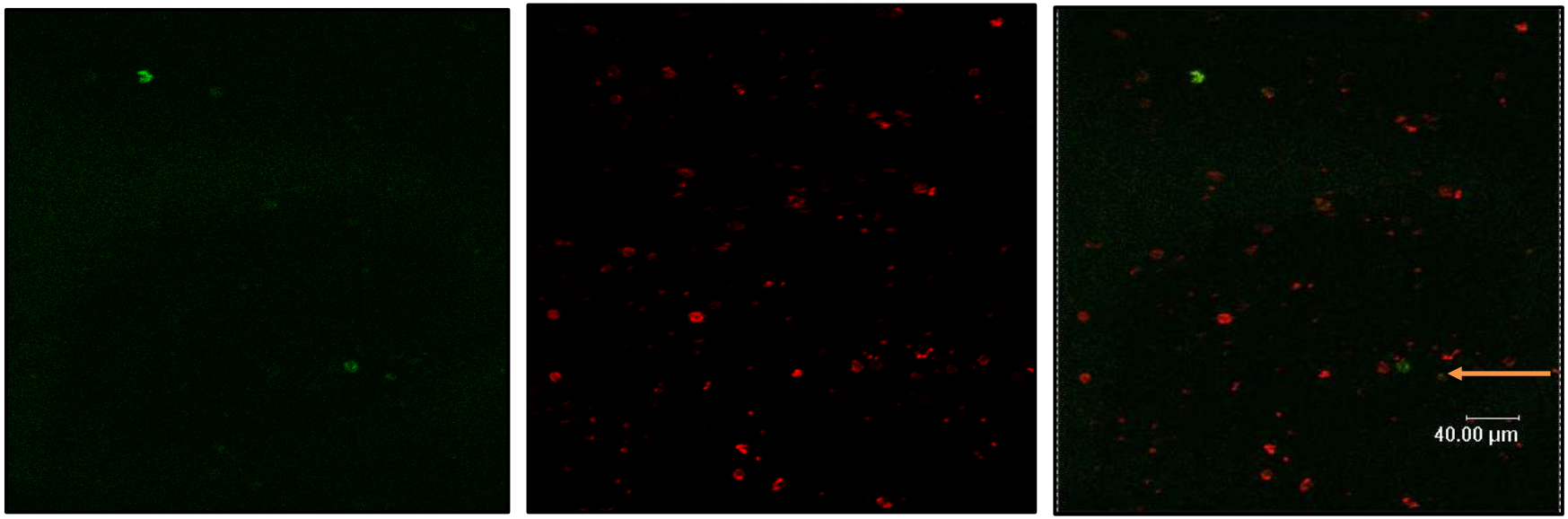

Figure 4.20 Confocal microscopy analysis of the interaction between DCs and liposomes in TST-positive individual.

Two immunofluorescence colours; in which the red colour represents DCs, meanwhile, the green colour illustrates the liposomes. Both images were overlaid to observe the uptake of liposomes by DCs as pointed by the orange arrow. Scale bars: 40 µm

Liposomes stained with FITC-488 nm | Dendritic cells stained with Rhodamine-Phalloidin-543 nm | Merge image

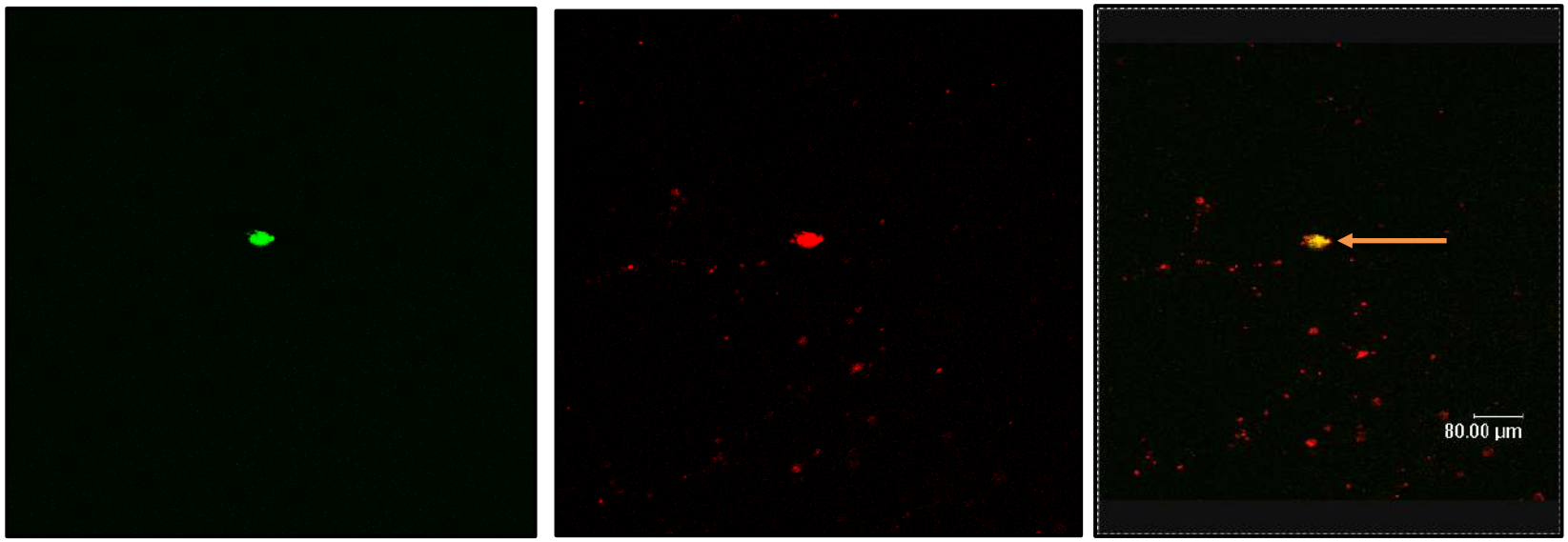

Figure 4.21 Confocal microscopy analysis of the interaction between DCs and liposomes in active pulmonary TB patient.

Two immunofluorescence colours; in which the red colour represents DCs, meanwhile, the green colour illustrating the liposomes. were overlaid to observe on the uptake of liposomes by DCs. Scale bars: 80 µm

CHAPTER 5

DISCUSSION

5.1 Sociodemographic factors of TST-negative individuals, TST-positive individuals, and active pulmonary tuberculosis patients

In this study, the demographical, clinical, and environmental characteristics of TST-negative individuals, TST-positive individuals, and active pulmonary TB patients were examined. The mean age of active pulmonary TB patients in the current study was 58 years old. This scenario could be assisted by the decreased amount and function of pDCs, which leads to the impairment of immune response against viral infections and microbes such as Mtb (Jing et al., 2009). In addition, the absence of TB clinical features might further complicate the diagnosis and prolonged the duration of starting treatment (Abbara et al., 2019). The epidemiology of TB showed the most significant trend of infection in the elderly, especially older men (Robins, 1953). Even in animal studies, it has been proven that the *in vivo* study supported the immune dysregulation by which the older groups are prone to infection due to their low production of T-cell responses (Rajagopalan, 2001). Hence, it has been demonstrated that those aged 65 years and over are the most potential group to be infected with TB (Caraux-Paz et al., 2021).

The highest number of participants among TST-positive individuals was shown to be in the middle-aged group. This event is highly related to their occupations with most of them serving as nurses and healthcare assistants. Hence, their job scope might require prolonged work duration with frequent in-contact with TB patients, increasing the risk of exposure to infectious diseases, including TB (Wardani et al., 2021). Previous studies exhibited similar findings of high TB risk prevalence among occupational healthcare workers (Peters et al., 2020). Sadaf et al.

reported an alarmingly high prevalence of latent TB infection among healthcare workers in tertiary care hospitals via IFN-γ assay (Sadaf et al., 2020). Therefore, healthcare workers have a double risk of contracting TB in comparison to the general population, indicating the high risk of bacterial exposure in healthcare settings (Liew et al., 2019).

There was no significant difference in the sex between all study cohorts, however, all the active pulmonary TB patients recruited were male. This might have been caused by the frequent and heavy intake of smoking with higher consumption of alcohol performed by men compared to women (Miller et al., 2021). Due to this reason, male TB patients were likely to be enrolled in this study instead of females. In addition to that postulation, other studies demonstrated that the treatment cost could contribute to the delayed diagnosis and treatment, owing to the responsibility of the man as the head of the family (van den Hof et al., 2010). The local transmission dynamics such as crowded, poorly ventilated, and nosocomial areas partially assist in the high number of pulmonary TB in men (Jiménez-Corona et al., 2006). These environmental lifestyles further contribute to high toxicity lung injury which are caused by the inhibition of TNF-α, leading to the suppression of immune cell function (Sopori et al., 1998; van den Hof et al., 2010).

The current study showed that the participants recruited were mainly comprised of Malays and Chinese, respectively. This condition was possibly associated with the highest population ethnicities in Kelantan, which consisted of 95.7% Malay, followed by 3.4% Chinese, 0.3% Indian, and 0.6% others. The outcome was concurrent with a previous TB survey performed from 2016 to 2020 in Terengganu. In their survey, the Malay race reported the highest survival and mortality rates upon treatment in comparison to the other races (Awang et al., 2022).

Herein, it can be concluded that the Malay race is the most prevalent to TB infection specifically in the East-Coast region.

Furthermore, this study also recruited a Thailand native as part of the active pulmonary TB patients. This scenario might have occurred due to the workforce of the migrated patient and the short distance of the border crossing Malaysia-Thailand which is located within the Rantau Panjang (Malaysia) and Sungai Golok (Thailand) immigrations. Consequently, there would be some outlooked cases of TB vaccination due to the illegal entry bypassing immigration, hence they could have been part of the reasoning that increase the number of TB infection. For instance, a previous statistic incidence of TB in 2014 had reported the highest number of TB cases in Selangor (65%) and Kelantan (25%), supporting the contribution of TB among local ethnics and foreign workers (Shahidatul-Adha et al., 2017). Due to this condition, a Thailand native had also been included as the subject in this study.

5.2 Clinical data on tuberculosis and its predisposing factor

The majority of the active pulmonary TB patients in this study reported their absence of TB history and zero contact with any TB-infected person or area. It could be assumed that this group was the primary TB, suggesting that they might inherently have low immunity levels. There was another possibility of this group might not be aware of their latency state, hence the Mtb infection was left untreated. Wetscherek et al. demonstrated primary TB group is presently common among adults in developed countries (Wetscherek et al., 2022). Therefore, the impaired immune system has been highly related to the multiplication of Mtb, which eventually leads to the induction of active TB (Brett et al., 2020).

However, some of the recruited participants in this study had developed a secondary or reactivated TB infection. It could have been due to household, non-household, or other environmental sources. Ai et al. supported the high risk of recently infected individuals reactivating Mtb within the past two years upon having household contact with active pulmonary TB (Ai et al., 2016). It has been evidenced by other studies that TB infection potential to reactivate with approximately 5 to 10% of latent TB individuals (Flynn & Chan, 2001). Hence, the predisposing environmental factors determined the state of TB infection within the recruited participants in this study.

During the sample collection of the current study, most of the active pulmonary TB patients were under the supervision of TB treatment of isoniazid, rifampicin, pyrazinamide, and ethambutol drugs. These drug regimens are preferable due to their efficiency in stopping Mtb growth (Burhan et al., 2013; Chideya et al., 2009). Besides, this short-course therapy has been implemented against TB infection for the last four decades (Dartois & Rubin, 2022).

Furthermore, it has been reported that most of the positive sputum smears had a positive culture with only a few cases of culture-negative results. Tostmann et al. demonstrated that the positive-cultured with negative sputum-smeared patients have a lower probability to infect others with a relative transmission rate of 0.24, which is inversely applied in those with both positive culture and sputum smear (Tostmann et al., 2008). This condition highlighted the importance of performing both tests to confirm the activation of TB infection and determine their levels of infectivity toward others (Finney et al., 2013).

In addition, the highest number of active smokers can be observed in active pulmonary TB in this study. This event could have been caused by the multiple

defects in immune cells such as monocytes, macrophages, mechanical disruption of cilia function, and other hormonal effects which eventually damaged the lungs (Alavi-Naini et al., 2012). The nicotine in tobacco smoke suppress the production of proinflammatory cytokines such as TNF-α, IFN-γ, IL-12, IL-8, IL-1, and IL-6, dampening the immune response against Mtb and impairing the capability of the body to control TB infection (Perriot et al., 2018). Passive smoking has been found to exhibit a high risk of active TB in child contacts of an index case (Altet et al., 1996). Hence, this supports the strong correlation between smoking increased risk of TB infection, TB mortality, recurrent TB, and treatment failure (Mahishale et al., 2015).

5.3 Liposomes production and characterization

This study used liposomes as the nanocarrier of interest in comparison to other nanotechnologies such as nanoparticles, micelles, polymers, and other substances. It is highly correlated with the unique properties offered by the liposomes such as reduced toxicity level, biodegradable, biocompatible, low, and affordable production cost, potent immunogenicity, and good targeting availability (De Jong & Borm, 2008; Inglut et al., 2020). The rapid development of liposomal adjuvant mechanisms and efficient drug delivery has been widely utilized in therapy and prevention for infectious diseases, including TB infection (Agger, 2016). For instance, cationic liposomes showed their extensive applications as both adjuvants and delivery systems in inducing proper immune responses against Mtb subunit vaccines (Farzad Khademi et al., 2018). In addition, Laterre et al. demonstrated a promising safety profile with good tolerability of CAL02 that is comprised of liposomes in a randomized human trial diagnosed with severe community-acquired

pneumonia (Laterre et al., 2019). Other studies reported that Epaxal is one of the approved clinical prophylactic vaccines formulated on the adjuvant of liposomes that have been used to treat Hepatitis A infection (Bulbake et al., 2017). This virosome-adjuvanted vaccine showed protection above 95% efficiency in adults receiving this booster, which could last up to 20 years (Bovier et al., 2002). Furthermore, the development of prolonged released amikacin (AMK) liposomes, Arikace, exhibited improved lung function in the nontuberculous mycobacterial lung disease of phase III clinical trial (Kwon & Koh, 2016). In short, these findings supported that liposomes are competent and more new formulations have the potential to succeed in clinical trials and eventually established standardized formulations for society.

M. smegmatis has been chosen as the source of liposomal production in the current study. This is due to the high genetic properties with over 90% similarities in comparison to Mtb such as the superoxide dismutase gene which assists in the survival of Mtb in macrophages (Liao et al., 2013; Shiloh & Champion, 2010). In addition, Ray et al. reported that *M. smegmatis* shares almost 76% homology with the orthologous EsxA effector protein from Mtb (Ray et al., 2019). This showed the high homology between the EsxA proteins from both *M. smegmatis* and Mtb could stimulate similar immune response upon T cells presentation, leading to an effective T cell activation and competent immune responses against TB infection (Hoang et al., 2013). Both fast-growing *M. smegmatis* and slow-growing *M. tuberculosis* demonstrated the presence of porins proteins in their cell walls which allows the diffusion of hydrophilic molecules, suggesting the potential for TB therapy (Singh et al., 2018). In addition, the culture period by *M. smegmatis* is very short and rapid, with two to three days of culturing and a doubling time of four hours (Wallace et al., 1988). Other studies showed that *M. smegmatis* has been commonly used as the

substitute model for highly pathogenic Mtb (Wei et al., 2000). For instance, Bohsali et al. reported *M. smegmatis* as one of the non-pathogenic mycobacteria groups that are unlikely to disseminate disease including in immunosuppressed individuals (Bohsali et al., 2010). Both species possess similar cell wall structures and components including mycolic acid, arabinogalactan, and peptidoglycan layers that allow interaction with immune cells and trigger desired immune responses (Baloni et al., 2014). Similarly, *in vitro* studies of Rv1954A knock-in *M. smegmatis* induced both innate and adaptive immune responses, supporting its capability as a TB vaccine candidate (Arora et al., 2020). Hence, these events supported the efficacy of *M. smegmatis* as a suitable surrogate model for the life-threatening Mtb.

In this study, the colony morphology of *M. smegmatis* was identified by using Ziehl-Neelsen (ZN) staining. This staining showed pinkish-red slender of *M. smegmatis* in bacilli shape. This observation is most likely due to the thick mycolic acid content and waxy structure in the cell wall, which resists the penetration of crystal violet staining and primarily takes the pink staining, instead (Yousif & Qasem, 2016). Pertaining to the cell wall, the cell envelope of *M. smegmatis* is comprised of strong immune stimulators such as mycolic acids, lipoarabinomann, arabinogalactan, and glycolipid layers (He & De Buck, 2010). Various studies had demonstrated the correlation between lipids of the cell envelope and pathogen resistance which leads to the modulation of immune responses (C. Ghazaei, 2018). The outer envelope lipids contribute to mycobacteria pathogenicity by mimicking the lipid distribution and antigen accessibility of the mycobacterial cell wall. This statement is supported by Garcia et al. who reported that liposomes derived from *M. smegmatis* have identical phosphatidylinositol mannosides (PIMs) with the Mtb polar glycolipids, stimulating *in vivo* specific IgG antibody response (de los Angeles

García et al., 2013). Hence, ZN staining offers a rapid, practical, and useful method to examine the presence of mycobacterium such as *M. smegmatis* in this study.

The dehydration-rehydration method was chosen among all the methods for liposomes production. This is mainly due to its simplicity and straightforward process, leading to a less time-consuming preparation with efficient entrapment of various ranges of materials (Kirby & Gregoriadis, 1984; Sharma et al., 2020). Apparently, French press cell extrusion and microfluidic techniques are the other alternatives for producing liposomes (Akbarzadeh et al., 2013). The French press cell extrusion method involves the gentle handling of unstable materials and can be simply executed within a short time duration (Dua et al., 2012). However, other studies reported that the maintenance of high temperatures and handling at relatively small working volumes as the major drawbacks of this method (Hamilton et al., 1980). On the contrary, the microfluidic method could easily possess scalable production of liposomes with a size range between 20-50 nm (Allen & Cullis, 2013). High solvent residues in the suspension of liposomes and large production scale are the main limitations of this method in comparison to the other conventional liposomes alternatives (X. Wang et al., 2018). These eventually supported that the dehydration-rehydration method has the upper hand in which it allows the control of particle size, which eventually produces small and unilamellar vesicles (Dua et al., 2012). Previous finding which established *M. smegmatis*-liposomes containing extracted glycolipids via a similar method possessed specific IgG antibody responses (de los Angeles García et al., 2013). Likewise, in a different mice study that derived the total lipid of *M. smegmatis* by using the same method, their result showed good *in vivo* protective capacity with potent immunogenicity against Mtb (Mat Luwi et al.,

2020). Therefore, the liposomes derived from the total lipid of *M. smegmatis* in this study are preferable to be prepared via the dehydration-rehydration method.

5.3.1 Field emission scanning electron microscopy

The characterization of produced liposomes via FESEM microscopy revealed its spherical shape with the size ranging between 20 to 135 nm. Hence, most of the currently produced liposomes was categorized as small unilamellar vesicles (SUVs) due to the range of diameter inferior within 100 nm (Bhupendra et al., 2015). This event was comparable with previously established findings performed by Luwi et al. that produced a similar size of liposomes, less than 100 nm (Mat Luwi et al., 2020). Another study reported that this hydration technique which synthesized 100 nm size range liposomes was suitable for direct drug delivery therapy (Sundar & Tirumkudulu, 2014). These findings suggested the capability of liposomes to improve the pharmacokinetics and pharmacodynamics of the drug, as well as increase the stability, shelf life, and release rate of the drug from the body (Betageri & Parsons, 1992; Yadav et al., 2017). Akbarzadeh et al. showed liposomes with a size less than 100 nm hold the advantage of possessing a higher rigidity (Akbarzadeh et al., 2013). The prolonged half-life of the current SUVs in comparison to the multilamellar liposomes (MLVs) promotes a low tendency to be recognized and internalized by the reticuloendothelial system (RES) (Immordino et al., 2006; Nisini et al., 2018). An increase by three- to four-fold encapsulation efficiency (EE) has been observed in a similar size range of liposomes produced via the dehydration-rehydration method compared to the mechanical lipid dispersion classical method (Aliño et al., 1990). Other finding found that the size range of liposomes which is similar to this current study could be efficiently taken up by DCs via clathrin-mediated endocytosis, leading to the stimulation of cell-mediated immune responses

(Deng et al., 2018; Xiang et al., 2006). Recently, an SUVs liposome-encapsulated antitubercular agent demonstrated a stable, high cellular uptake with improved EE as opposed to the free antitubercular drug (Kósa et al., 2021). Therefore, FESEM analysis mainly produces high-resolution images of the sample's surface, allowing further description of its shape and size. The results obtained supported the particular size of 100 nm liposomes produced by the dehydration-rehydration method in this study.

Summarily, the physicochemical properties of liposomes assist in their utilization as a vaccine adjuvant-delivery system for distinctive clinical therapies, particularly against infectious diseases like TB infection. The pivotal criterion such as lipid composition manufacturing process, and physical size, collectively influence the stimulatory effect. Compared to the previous studies, the liposomes produced in this study have the advantage of utilizing fast-growing and non-pathogenic mycobacteria that resulted in a small-sized range of liposomes. Herein, this liposome could be a potential adjuvant, drug carrier, and vaccine candidate is suitable for human-scale testing.

5.4 Surface marker expression of dendritic cells between TST-negative individuals, TST-positive individuals, and active pulmonary tuberculosis patients upon exposure to liposomes by flow cytometry

5.4.1 Percentage of dendritic cells and their subsets, myeloid dendritic cells and plasmacytoid dendritic cells

In the present study, it was found that the percentage of DCs and their subsets, mDCs, and pDCs, were the lowest in active pulmonary TB patients compared with TST-negative and TST-positive individuals. This might be due to the suppression action of Mtb which modulates the DCs function (Zhang et al., 2020).

For instance, Su et al. reported that the mannosylated glycoprotein of Mtb, Rv1016c, could impair the maturation of DCs via TLR2/STAT/SOC3 pathway (Su et al., 2019). The absolute numbers of DCs subsets have been associated with prolonged TB, anti-tubercular drugs (ATD) effect, and lymphocyte immune responses, and are commonly used as an effective diagnostic biomarker for active pulmonary TB screening (Lu et al., 2017). This ATD eventually reduce the bacterial load and inflammation, impacting the overall immune responses including the function of DCs. Previous study showed the first-line anti-tubercular drugs such as isoniazid, rifampicin, ethambutol, and pyrazinamide functioned by inhibiting the DNA-dependent RNA polymerase, thus suppressing the cell wall, protein, and nucleic acid synthesis of Mtb (Sotgiu et al., 2015). The result of the current finding was comparable with another study in terms of the specific and significant decrease in the absolute number of circulating DCs in patients with active TB compared to healthy donors (Lichtner et al., 2006). Similarly, a significant decrease in the number of DCs has been demonstrated in the tuberculous granulomas via immunohistochemical analyses (Uehira et al., 2002). Hence, these implied DCs as one of the major innate and adaptive immune cells that are more likely to be infected by Mtb.

In addition, active pulmonary TB patients showed an inverse pattern of having higher circulating levels of mDCs but with lower pDCs counts when compared to the TST-negative and TST-positive individuals. This event could perhaps be due to the anti-tuberculous treatment performed via directly observed treatment short course (DOTS), suggesting the impact of active disease on circulating DCs subsets (Gupta et al., 2010). However, the mechanism action varies and highly relies on the drugs prescribed during the DOTS therapy. It is important to note that the percentage of pDCs in healthy is normally constituted less than 0.5%

which correlates with the result of this study, supporting that patients with TB tend to have lower pDCs counts than mDCs as expected (Ye et al., 2020). These observations concurrent with findings of pDCs showed the most significant reduction counts in both pulmonary and extrapulmonary TB (Lichtner et al., 2006). Mendelson et al. reported patients with pleural TB characterized an increased number of mDCs than the healthy controls (Mendelson et al., 2006). Interestingly, other studies exhibited contradicting results on the accumulation of DCs in the tuberculous granuloma which resulted in lower absolute counts of mDCs in active pulmonary TB patients than the healthy controls (Lu et al., 2017). These findings reflect the good response of DCs upon being treated with the available anti-TB drugs. The mDCs mainly focus on modulating adaptive immune response, while the pDCs influence by balancing both pro-inflammatory and anti-inflammatory responses. Therefore, it is essential to compare the subsets of DCs to gain a further understanding of how these specific cell subsets contribute to TB progression, and immunity, as well as for future potential treatment strategies.

The exposure of liposomes to DCs and their subsets demonstrated an improved absolute total number of DCs, except for the mDCs counts in active pulmonary TB patients. This result highlights that liposomes exhibited their immune-enhancing activities to the antigens through the elevation of antigen uptake by the DCs mechanism which in turn activates the immune responses (Takahashi et al., 2017). Apart from neutral sources of liposomes, cationic liposomes are potent for direct activation of DCs without the addition of adjuvants in cancer vaccination (Nakanishi et al., 1999). Other studies on cationic liposomes containing trimethyl ammonium propane showed a high percentage of both DCs subsets through *in vitro* monocyte-derived human DCs and murine bone marrow-derived DCs (BMDCs)

(Foged et al., 2004). Therefore, suggesting the critical role of liposomes as an efficient adjuvant.

5.4.2 Assessment of dendritic cells and their co-stimulatory markers

In this study, the exposure of liposomes in active pulmonary TB exhibited a significantly diminished expression of HLA-DR, accompanied by the enhanced level of $CD11c^{-}CD123^{+}$pDCs and reduced CD86 expression although the trends were not significant when compared to TST-negative and TST-positive individuals. This event may be due to the decreased number of mDCs and pDCs in active pulmonary TB patients in which only pDCs will be recovered upon treatment (Lu et al., 2017; Uehira et al., 2002). The independent role of DCs is associated with the deficiency of DCs, where DCs serve as *in vivo* reservoirs for Mtb during the latent state, meanwhile, during the active TB state, this cell is highly prone to be destructed via apoptosis (Lichtner et al., 2006). In addition, the increased demand for the recirculation of cells to the affected organs could potentially contribute to the decreased number of peripheral blood DCs (Lichtner et al., 2006). Multiple studies had supported the current finding. For instance, diminished expressions of HLA-DR and CD1c antigen-presenting cells alongside low co-stimulatory molecules CD86, CD40, CD150, CD28, and CD152 have been observed in the lung cells in patients with TB compared with non-TB controls (Flores-Batista et al., 2007). In another infectious disease such as Hepatitis C Virus (HCV), the level of HLA-DR and CD86 expression were lower in mDCs of HCV-infected patients than the healthy donor (Averill et al., 2007). Interestingly, Lu et al. reported contradicted outcomes of low HLA-DR and CD80 expressions with a high CD86 level in the peripheral blood of active pulmonary TB patients compared with healthy controls (Lu et al., 2017). Hence, the current result suggested the potential of liposomes in DCs engage in the

immunological changes of TB pathogenesis via the improved HLA-DR presentation and CD123 shown.

The exposure of liposomes in active pulmonary TB patients improved HLA-DR antigen presentation with a significant increase of co-stimulatory activation marker, CD86 when compared to the positive control group. This event portrayed the immunostimulatory effects of liposomes due to their localization which is located at the cell surface and their properties as a component of the cell wall of mycobacteria (García Mde et al., 2014; Homhuan et al., 2007). Their unique cell component such as lipids, proteins, and genetic material has the potential to act as adjuvant or antigen marker for the development of subunit vaccines (De Serrano & Burkhart, 2017). For instance, mannophosphoino-sitides (PIMs) antigen of mycobacteria exhibited double stimulation of immune responses upon being encapsulated with liposomes in comparison to the single glycolipid (Singh & Khuller, 1993a). Previous studies characterized high HLA-DR, CD86, and CD80 levels upon the interaction of their DCs with the liposomes derived from the total polar lipid of both non-pathogenic bacteria *M. smegmatis* and *Leptospira biflexa* serovar Potac (Faisal et al., 2011). The humoral and cell-mediated immune responses observed in both liposomes were eventually stronger in comparison to the conventional liposomes and conventional aluminum hydroxide adjuvant (Faisal et al., 2011). Similarly, liposomes derived from *M. smegmatis* supported the immune activation of BMDCs through the upregulated expression of CD86 in the Mtb mice model (Mat Luwi et al., 2020). In a different study, Sprott et al. reported that liposomes extracted from the total polar lipids of *M. bovis* BCG enhanced the expression of CD80, CD86, and CD40 co-stimulatory markers, supporting the efficiency of lipids in activating DCs (Sprott et al., 2004). It can be deduced that liposomes formulation of mycobacterial lipids is

highly reliable to initiate efficient immune responses in comparison to other types of liposomes.

Although the differences were not significant, an increase in the HLA-DR expression with a stagnant level of CD86 has been observed in the presence of liposomes within the active pulmonary TB patients in comparison to the negative control. This could potentially associate with DCs malfunction via the interference of 19-kDa Mtb lipoprotein, which results in rapid DCs maturation and inhibition of MHC class II antigen presentation to effector cells (Baena & Porcelli, 2009; Chang et al., 2005; Hava et al., 2008). Therefore, the liposomes may have produced damage to the cells, possibly triggering toxicity effects but further confirmation tests are highly required to verify this proposition. A study revealed that the cytotoxicity effect of the liposomes to the cells might be associated with the dosage or amount of lipid presented (Lechanteur et al., 2018; Romøren et al., 2004). The toxicity effects of the cationic liposomes towards normal cells could possibly cause by its electrostatic interaction, leading to cell damage (He & Tang, 2018). Cong et al. had further supported in which cationic liposomes/DNA complexes (CLN/DNA) exhibited cytotoxic effects to the tumour cells alongside with the enhancement of tumour cell lysis (Cong et al., 2020). Interestingly, high-density octaarginine-modified liposomes promoted significantly enhanced levels of MHC class II, CD80, and CD86 co-stimulatory markers (Homhuan et al., 2007). This is further supported by previous murine models in which the current *M. smegmatis* liposomes demonstrated high expressions of MHC II and CD86 (Mat Luwi et al., 2020). The liposomes in both TST-negative and TST-positive individuals showed no significant differences in the presentation marker HLA-DR in comparison to the positive and negative controls. However, their CD86 expression upon exposure to liposomes was

increased significantly in comparison to the positive control. This current result is consistent with the outcomes shown in the active pulmonary TB patient group in which the liposomes trigger maturation and activation of DCs.

Lipopolysaccharide (LPS) from *E. coli* has been an efficient stimulator for CD80 and CD86 markers (Bartheldyová et al., 2019). On the contrary, the present study showed that the presentation and activation of DCs were downmodulated in bacterial LPS stimulation. This event is to be possibly caused by the decreased antigen export to the cytosol which can be induced by high doses of pure LPS (Alloatti et al., 2016; Gros & Amigorena, 2019). The result of the current finding was comparable with previous studies which demonstrated low expression of cross-presentation in the late phases DCs in response to LPS (Gil-Torregrosa et al., 2004). The downregulation of LPS is promoted by the transcription factor EB gene which leads to endosomal acidification and antigen degradation (Samie & Cresswell, 2015). Interestingly, other study showed contradicting outcomes with a high expression by LPS which further downregulate CD11c and CD11b levels of DCs via cellular apoptosis (Griffiths et al., 2014). Thus, the volume of LPS needs to be well determined as it plays a crucial role in stimulating the desired immune response.

Taken together, the current study supported the interplay between liposomes and immune responses via specific targeting of DCs. The observations presented could be a potential guide when choosing the appropriate liposomes as drug carrier or vaccine adjuvant. Indeed, the mycobacteria cell envelope lipid showed potential in modulating strong humoral and cell-mediated immune responses that are almost comparable to the gold standard BCG vaccine.

5.5 Cytokine secretion

This study found a significantly increased concentration of IFN-γ in DCs of active pulmonary TB patients upon exposure to liposomes compared to all groups. This observation could be due to the activation of immune cells and the immune responses triggered upon exposure by liposomes. IFN-γ cytokine has been preferentially known as a relevant marker that stimulate the protective cell-mediated immune response (Agger & Andersen, 2001). In addition, IL-12p70 might have promotes the secretion of IFN-γ which is required to control the growth of Mtb infection (Khader et al., 2005). High induction of IFN-γ cytokine with increased IgG2b titers via the administration of cationic DDA-TDB liposomes has been reported in the mice model immunized with Mtb fusion antigen Ag85B-ESAT-6 (Davidsen et al., 2005). A cationic mycobacterial monomycolated glycerol liposomes was found to rise prominent Th1 response through IFN-γ production in tuberculosis-infected mice (C. S. Andersen et al., 2009). Shamshiri et al. demonstrated a comparable finding in which PEGylated liposomes induced high secretion of IFN-γ response to the tumor area in comparison to the non-PEGylated liposomes of colon cancer *in vivo* (Kateh Shamshiri et al., 2021). Besides, a galactosylated liposomes study showed significantly increased concentration of IFN-γ, IL-4, and other cytokines, indicating liposomes as a dendritic cell-targeted mucosal vaccine against tumors (Jiang et al., 2015). However, there has been another study claiming that liposomes with a size >225 nm tend to stimulate Th1 responses due to the elevated levels of IgG2a and IFN-γ. Meanwhile, smaller liposomes <155nm induce Th2 responses from the high secretion of IL-5 and IgG1 (Brewer et al., 1998). Their statement is contradicting the current study as our small liposomes stimulate high IFN-γ secretion, suggesting the composition of liposomes could be one of the factors

that need to be taken into consideration. Yet, it can be clearly proposed that liposome stimulation could increase the concentration of IFN-γ cytokine.

Furthermore, the secretion level of IL-12p70 in the DCs of active pulmonary TB patients upon exposure to liposomes was significantly higher than TST-negative and TST-positive individuals. It has been found that the IL-12p70 level in disseminated TB (such as pulmonary TB) was greater compared to the healthy control. Essone et al. supported latent TB in healthcare workers that nursed TB patients enhanced the secretion of pro-inflammatory cytokines such as IL-12p70, IL-6, IFN-γ, and IL-8 (Essone et al., 2019). The IL-12p70 cytokine is one of the six-cytokines that primarily differentiate active infection from latent TB (S. Wang et al., 2018). Although the crucial role of IL-12 in stimulating Th1 and cytotoxic T lymphocytes has been emphasized, yet low amounts of IL-12 were secreted in DCs via TLR signaling (He et al., 2015). Hence, the current study positively identified the potential of liposomes derived from *M. smegmatis* as an efficient adjuvant in assisting the robust production of IL-12p70 to improve the presentation by DCs and induce Th1 response for host defense against Mtb infection (Keegan et al., 2018). Garu et al. showed that *in vivo* dendritic cell-targeting liposomal DNA vaccine succeeded in increasing the secretion of IL-12p70, IL-12, and TNF-α (Garu et al., 2016). The production of IL-12p70 cytokine was consistent with another study by liposomes coated with α1-3, α1-6-mannotriose neo glycolipid, leading to the stimulation of Th1 response (Matsuoka et al., 2019). These investigations may provide inputs on the liposome formulation as a promising candidate that triggers IL-12p70 cytokine release.

In this current study, the concentration of IL-4 was significantly increased in the DCs of active pulmonary TB patients exposed to liposomes when compared to

the DCs of TST-positive individuals exposed to liposomes. The secretion of IL-4 has been the hallmark of Th2 which is essential for humoral immune responses, whereas IFN-γ secretion that associated with Th1 development is crucial for cellular immune responses (Chen et al., 2004). This reflects the potential of liposomes to enhance the number of $CD4^+$ and $CD8^+$ T cells secreting IL-4 or IFN-γ cytokine (Elser et al., 2002; Mao et al., 2022). However, there has been controversy surrounding the role of IL-4 cytokine in TB progression. Ordway et al. reported increased IL-4 production in TB patients, which was in contradiction with the study performed by Nie et al. that showed diminished or no statistically significant changes in the IL-4 expression (Nie et al., 2020; Ordway et al., 2005). This suggests that IL-4 may not necessarily skew a Th cell phenotype due to its presence in all conditions such as in the healthy control, latent TB, and active TB (Veenstra et al., 2008; Verbon et al., 1999). It has also been studied that IL-4 could suppress IFN-γ and activate alternative macrophage activation, that is distinct from the classical Th1 activation, allowing the elimination of Mtb by macrophage (Gordon, 2003). Recently, cationic liposomes comprised of cell wall surface protein of *Candida albicans* displayed good immunogenic performance via the elevated concentration levels of IL-4, IL-17, and IL-10 than control groups (Carneiro et al., 2015). Huang et al. demonstrated consistent outcomes in which the production level of IL-4 was significantly high in the natural polymer-based liposomes adjuvant from day 7 to day 42, in comparison to the other controls (Huang et al., 2016). The performance of the TB subunit Rv0572c vaccine that has been emulsified into liposomes triggered a rather limited concentration of IL-4, a Th2 humoral immune response (Mao et al., 2022). These studies proposed the competency of liposomes to regulate the concentration of IL-4 cytokine.

Herein, the present study concluded that liposomes derived from *M. smegmatis* has the capability to induce Th1 and Th2 responses via the secreted levels of IL-4, IL-12p70, and IFN-γ cytokines which were mainly observed in active pulmonary TB patients. This event correlates with the stimulation of DCs activation marker, CD86.

5.6 Association study

In the association study, it has been found that the trends between surface marker, CD86, and all cytokines were negatively associated, particularly in the DCs of TST-negative individuals and active pulmonary TB patients upon the exposure to liposome. This emphasizes that the activation marker, CD86, is primarily influenced by the presence of pathogens, rather than being directly dependent on the secretion of cytokines. These conditions might potentially be caused by the potent immunostimulatory effects of the liposomes which secretes cytokine even in the settings of DCs being dysfunctional. Schmitz et al. supported this statement when they found that the secretion of IL-4 can also be stimulated by non-Th cells and not only by DCs, although further studies were required to distinguish them (Schmitz et al., 1994). In a negatively charged liposomes study, the production of IL-12 was increased via the synergy between CD40L of 42-6A cells and CD40 on macrophage/DCs (Yotsumoto et al., 2004). However, another findings reported contradicting outcomes in which the activation of DCs was dependent on cytokines production, particularly upon exposure to liposomes. For instance, phosphatidylserine (PS) liposomes mimicked the apoptotic cells by suppressing the level of CD86 and IL-12 cytokine in murine models (Shi et al., 2007). In short, these

observations indicate the independency of IFN-γ, IL-12p70, and IL-4 secretions upon being exposed to liposomes.

5.7 Uptake and encapsulation of liposomes by dendritic cells

5.7.1 Field emission scanning electron microscopy

In this study, FESEM analysis has been used to compare the uptake, size, and shape of DCs in distinct conditions within all groups. This finding presented may contribute new ideas and knowledge on the structural differences of DCs between active pulmonary TB patients with TST-positive and TST-negative individuals.

The shape of DCs without any stimulators characterized the development of dendrites which was only presented in active pulmonary TB patients, contrasting to the DCs of TST-negative and TST-positive individuals. This event most likely suggested that the DCs of active pulmonary TB patients have undergone maturation and activation potentially due to Mtb infection. The interaction between the proline-glutamate (PE) protein of Mtb and DCs have shown to possess Th-1 polarizing potential via DCs maturation (Kim et al., 2016). The present finding was comparable to animal studies which illustrated rounded to oval cell bodies with short cell processes of matured DCs in the lungs of rabbit models (Mokhtar & Hussein, 2019). The morphology of DCs in infected-TB guinea pigs exhibited a similar characterization of having dendrites protrusions (Dascher et al., 2002). Indeed, the structure of normal healthy DCs before sustaining the differentiation process is characterized as round-shaped with a smooth surface.

In TST-positive individuals, their circular smooth DCs showed that the lumps on the surface of cells not as apparent as the DCs of TST-negative individuals. The current findings potentially suggest that the Mtb could have modulated the DCs by

masking its presence, impairing the specific function of DCs to stimulate specific T cells (Geijtenbeek et al., 2003). Several studies demonstrated that the interactions of Mtb with DC-SIGN might have suppressed the TNFkB activation, thus inhibit the formation of dendrites (Mihret, 2012). The Mtb specifically targets DC-SIGN via its cell wall component ManLAM to prevent the maturation of DCs, leading to the secretion of anti-inflammatory cytokine, IL-10 (Geijtenbeek et al., 2003). However, the occurrence of this scenario could also perhaps be caused by the latency associated with Mtb proteins Hip1 and Acr (Mayito et al., 2019). These events correlated with the early stage of non-differentiated cells in TST-negative individuals which tends to appear in a round and smooth formation with the presence of a large nucleus and active chromatin (Soumelis & Liu, 2006). Grouard et al. illustrated similar microscopic examinations in which their human freshly isolated pDCs were of round-shaped cells with short processes presented on the surfaces, resembling the plasma cells as their name indicates (Dalod et al., 2014; Grouard et al., 1997). The ridge circular morphology could be observed through *in vivo* bone-marrow-derived immature DCs (Kim & Kim, 2019). These studies eventually deduced the structural changes of DCs in the presence of Mtb.

In contrast, active pulmonary TB patients visualized a rather circular DCs with the presence of short dendrites upon LPS stimulation unlike the extended dendrites protrusions illustrated in TST-negative and TST-positive individuals. It can be suggested that the LPS unable to induce the desired antigen presentation to T cells due to the dysfunctionality of DCs. The outcomes of the current study have been potentially supported by cancerous studies which reported that accumulation of high lipid content in DCs could impair its function and further inhibit the stimulation of T-cells (Herber et al., 2010). Zanoni et al. proposed that LPS induction might have

triggered the apoptotic death of differentiated DCs via the activated nuclear factor of activated T cells (NFAT) (Zanoni et al., 2009). Meanwhile, in a normal condition such as TST-negative individual, the LPS is detected by TLR4 which serves as a signal for DCs migration and stimulate adaptive immune responses (Gröbner et al., 2014). Other studies exhibited multiple microvillus-like cell projections via the mature DCs derived from human cord blood (Neumüller et al., 2016). The ultra-structure of mature mouse bone-marrow DCs which were exposed to LPS presented remarkably long with increased branch protrusions of dendrite morphologies (Zeng et al., 2012). Some of the LPS-induced BDMCs eventually have bigger protrusions with rough surfaces in comparison to the control (Xing et al., 2011). Thus, the formation of dendrites convinced the maturation and activation of DCs which occur due to the presence of LPS as stimulant.

The current FESEM findings supported the uptake of DCs via flow analysis in which the liposomes may have a cytotoxic effect on DCs of active pulmonary TB patients, inducing cell death apoptosis. This condition could potentially be due to the presence of glycolipids in the current liposomes that may inhibit DCs via promising receptors such as DC-SIGN or mannose receptors (Pouget et al., 2021). Earlier *in vitro* herpes simplex virus type 1 studies revealed increased cytotoxicity levels upon the exposure of liposomal peptide-tripalmitoyl-S-glyeryl cysteinyl exposure on DCs (Nair et al., 1993). Hiromatsu et al. demonstrated similar cytotoxic activities against BMDCs of guinea pigs immunized with Mtb total lipid Ag, supporting the outcomes of the present study (Hiromatsu et al., 2002). Herein, the functional mycobacteria liposomes could have induced apoptosis specifically in active patients with TB infection.

However, the ability of DCs to uptake the liposomes can be observed in the DCs of TST-positive and TST-negative individuals, which possessed numerous long extended dendrites with clearer internalization of liposomes on their surface. This scenario emphasize that the liposome has been recognize and taken up by DCs, leading to its activation. Besides, this result correlates with the obvious signaling uptake of liposomes by DCs via confocal analysis which was discussed latter in this study. Hence, suggesting its crucial play in TB pathogenesis and stimulation of protective immunity against Mtb infection (Borrero et al., 2013). This present finding is similar to the animal study that uses liposomes derived from *M. smegmatis*, in which the liposomes are localized on the surface of bone marrow derived DCs, emphasizing on the liposomal uptake by BMDCs (Mat Luwi et al., 2020). Previous *in vivo* cervical carcinoma studies demonstrated efficient delivery of human papillomavirus (PHV)-E7 epitope antigen with improved anti-tumor immune responses by cationic liposomes (Vangasseri et al., 2006). Boks et al. observed consistent liposomal binding and uptake by specific DC targeting via their glycan Lewis X-modified liposomes (Boks et al., 2015). For these reasons, liposomes have been reported to inherent efficient immunogenic responses.

In addition, the current study emphasizes the uptake of liposomes by DCs through the comparison with the normal-sized scattered liposomes, suggesting that certain liposomes may not be uptake by the immature DCs. Previous studies supported the potential of DCs to uptake the liposomes, although there could have been a possibility of other cells like macrophages that enhance stronger uptake in comparison to DCs (Vanbever et al., 2019). Kaur et al. reported that their liposomes formulation with a size range of 20-100 nm has potential to influence the uptake by DCs (Kaur, 2011). Other cationic liposomes comprise of dimethyl-

dioctadecylammonium (DDA) and trehalose 6,6-dibehenate (TDB) showed a significantly increased capacity of inducing immune responses when compared to the large MLVs, acknowledging liposomes as potent vaccine adjuvants (Milicic et al., 2012). Therefore, recommending the adjuvant properties provided by liposomes in triggering the maturation of DCs and subsequently signaling the production of essential co-stimulatory molecules for T-cell priming (Gardner & Ruffell, 2016).

Taken together, *M. smegmatis* liposomes endow as highly competent adjuvant and carrier to potentiate desired immune responses. Its uptake by DCs, further supported its capability as a potential candidate for the modern vaccine formulation in infectious diseases, such as TB infection.

5.7.2 Confocal microscopy

The uptake of liposomes by DCs has been assisted by the verification of the confocal analysis. In the current study, the FITC-conjugated liposomes of all study cohorts were observed to be localized inside the DCs. This interaction could have been due to the structure of liposomes which comprised hydrophobic and hydrophilic phospholipids that increased cellular uptake (Zhang et al., 2019). Maji et al. study promote current findings with their confocal analysis illustrating the efficient uptake of cationic liposomes by DCs in *Leishmania* infection (Maji et al., 2016). The co-localization of phosphatidylserine liposomes loaded with Mtb antigen has been monitored to be entrapped by DCs in both animal and human models (Diogo et al., 2019). Besides, previous *in vitro* performance of lipid-PGLA hybrid nanoparticles exhibited similar outcomes, recommending the enhanced uptake of nanoparticles by DCs (Hu et al., 2014). These observations supported the intracellular localization of liposomes by DCs.

The DCs in active pulmonary TB patients illustrates a rather strong fluorescent formation than the weak fluorescent in TST-positive individuals upon being exposed to liposomes. Meanwhile, the bright fluorescent signal but with a lesser number of cells has been observed in the active pulmonary TB patients in comparison to the TST-negative individuals. These scenarios could potentially mediate by the adsorption of liposomes onto the surface of DCs which leads to efficient endocytosis (Miller et al., 1998). Early confocal studies revealed the uptake of mannosylated and histidylated liposomes via clathrin-mediated endocytosis as shown in their murine DC2.4 line (Perche et al., 2011). Takahashi et al. supported the current evidence in which their cationic liposomes showed enhanced uptake of ovalbumin by murine DCs cell line, DC2.4 through the clathrin- and caveolae—independent but lipid-raft-dependent endocytic pathways (Takahashi et al., 2017). In a different study that uses liposomes coated nanodiamonds, their human primary DCs exhibited the uptake of nanodiamonds that localized inside the cells (Nie et al., 2022). Interestingly, the present finding is comparable to Foged et al. study in which their cationic liposomes that comprised trimethyl ammonium propane detected strong intracellular signals in the high percentage subsets of both murine bone marrow-derived DCs and monocytes-derived human DCs (Foged et al., 2004). Indeed, the internalization of liposomes by DCs is crucial to trigger specific immune responses, supporting its adjuvant effect.

In short, the internalization process of liposomes derived from *M. smegmatis* via confocal microscopy provided additional insight into the uptake process and activation of DCs. The interaction between liposomes and the cell membrane highly depends on the nature formulation such as size, shape, and surface properties. Thus, the liposomes produced in this study succeeded to be up taken by DCs.

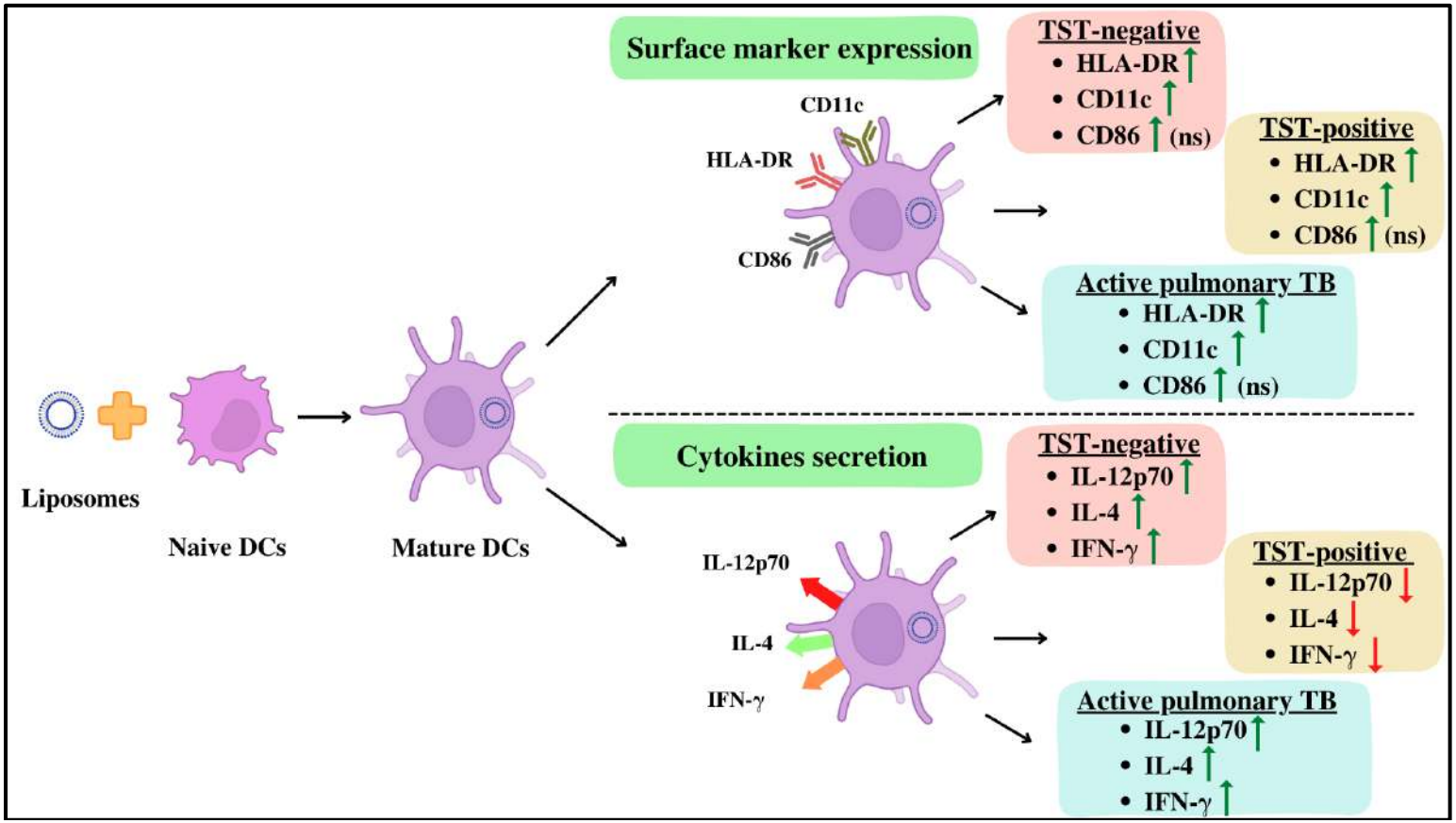

Figure 5.1 Overview of findings in the study.

The exposure of liposomes to DCs of TST-negative individuals, TST-positive individuals, and active pulmonary TB patients regulate the activation of cells and assists the uptake of the liposomes. (ns=not significant)

CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATIONS

6.1 Limitation of the study and recommendations for future research

Several limitations have been acknowledged in this current finding. Firstly, this study only measured the size of liposomes by using the established FESEM method. In this regard, the ZetaSizer method could be used for further characterization of liposomes due to the highly accurate and repetitive size measurement, providing more reliable results. This method was unable to be performed due to the fragility of liposomes. Next, the uptake of liposomes by DCs has been observed via a confocal microscope which is only visualized from one plane focus. The current microscope was unsuitable for the analysis of these fragile liposomes. Hence, it would be advantageous to perform a Z-stack analysis which could provide three-dimensional images captured at different angles. Furthermore, this study could have provided the identification of lipids present in current liposomes. Thin layer chromatography (TLC) can isolate and detect the purity of lipid compounds that exist and eventually provide additional understanding of liposomes. However, the standardized TLC method for the current liposomes has not met the desired expectation, thus further optimization tests are still required. Besides, it was preferable to test the stability of the current liposomes. This analysis was not performed in the current study since the liposomes can easily evaporate during transportation. Both foster resonance energy transfer (FRET) and epifluorescence microscopy could have given insight into the physical stability of the present liposomes.

In addition, this study initially proposed to recruit n=138, with 46 participants for each group. However, this sample size has been reduced during the period of sample collection due to the ongoing COVID-19 pandemic and the restriction of

movement orders. These conditions had put the study on a halt due to the prohibited entry into the ward and clinic. Furthermore, the male and Malay participants have been enrolled as active pulmonary TB patients in this study. This condition mainly occurred due to the hospital placement in which HUSM received mostly male infected-TB patients, meanwhile, the female activated-TB will be receiving their treatments in HRPZ. It would have been advantageous to recruit females, and other races such as Chinese or Indians to reduce the bias of gender and race in the performance of the study.

This current study was mainly investigating the uptake of DCs by liposomes. It could have been highly promising to confirm this interaction via the inhibition study. These inhibitors could verify that the illustrated uptake study was not attributed to the cell death mechanisms. Although this method may not be performed by this study, it is currently being tested in a continuation study. Cytotoxicity study is also recommended for future study to measure its potential toxicity effect towards cell that may cause cell death. RT-qPCR can be performed to further validate the cytokine expression of IFN-γ, IL-4, and IL-12p70 within the molecular level. This method can provide the relative gene expression of targeted DCs upon exposure to liposomes. The western blotting analysis is also proposed to identify the signaling pathway of DCs stimulated by liposomes, supporting further understanding of the mechanism involved. More cell surface markers to identify specific DCs such as CD80, CD103, CD83, etc. are highly encouraged for future works. Lastly, the current study only focuses on the effect of liposomes upon being exposed to different groups. Indeed, the duration of the study needs to be further extended at different time-point to ensure the safety and adverse effects of current liposomes.

6.2 Conclusion

The SUVs liposomes derived from total lipid *M. smegmatis* have been successfully produced, equivalent to its spherical shape formation with size ranging between 20 to 135 nm analysed. Upon the isolation of human PBMCs, the interaction between liposomes and DCs was investigated through numerical and visual observations. The immune profile of mean percentage DCs in active pulmonary TB patients in this study showed the downregulation of cell counts compared to the TST-negative and TST-positive individuals. However, the presence of liposomes has been shown to induce stimulatory responses effectively. The presentation of HLA-DR expression and CD86 co-stimulatory marker was eventually improved, alongside the increased concentration levels of IL-12p70, IFN-γ, and IL-4 in active pulmonary TB patients. The presence of liposomes on the surface of DCs and the detection of fluorescence signal showcased the uptake of liposomes upon the immune activation of DCs. Herein, the current study represents the crucial role of *M. smegmatis* liposomes as a potential adjuvant, carrier, or vaccine that targets DCs through the stimulation of both humoral and cell-mediated immune responses. This suggests that liposomes from mycobacterial lipids as an efficient vaccine candidate and immunotherapeutic agent for TB infection.

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APPENDICES

Appendix A

Ethical approval from the Human Research Ethics Committee (HREC) USM.

Jawatankuasa Etika
Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

20th May 2019

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JEPeM Code : USM/JEPeM/19010087
Protocol Title : Deciphering the Mechanisms of Cellular Uptake of Natural Liposomes Derived from *Mycobacterium Smegmatis* (Liposomes-Msmeg) as a Lipid Based Vaccine by DCs of Human Peripheral Blood Monocytes (PBMC) of Healthy and Patient with TB.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/19010087**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **20th May 2019** until **19th May 2020.**

Study Site: Hospital Universiti Sains Malaysia.

The following researchers also involve in this study:

1. Prof. Dr. Norazmi Mohd Nor
2. Prof. Dr. Armando Acosta
3. Prof. Dr. Maria Elena Sarmiento Garcia San Miguel
4. Assoc. Prof. Dr. Siti Suraiya Md Noor
5. Dr. Rohimah Mohamud
6. Dr. Tan Hern Tze
7. Dr. Alwi Muhd Besari @ Hashim

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient/Participant Information Sheet and Consent Form (English version)
2. Patient/Participant Information Sheet and Consent Form (Malay version)
3. Study Form

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2019: Continuing Review Application Form.**

JEPeM
JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA

Appendix B

Participant information sheet and consent form

RESEARCH INFORMATION (PATIENT)

Research title:	**Deciphering the mechanisms of cellular uptake of natural liposomes derived from *Mycobacterium smegmatis* (liposomes-Msmeg) as a lipid-based vaccine by DCs of human peripheral blood monocytes (PBMCs) of healthy and patients with TB** **(Merungkai mekanisma pengambilan liposom yang berasal daripada mikobakteria sebagai vaksin oleh sel dendritik daripada sel darah putih bagi individu yang sihat dan pesakit TB)**
Main researcher:	**Dr. Ramlah Kadir**
Co-Researchers:	**Professor Dr. Norazmi Mohd Nor** **Professor Dr. Armando Acosta** **Professor Dr. Maria Elena Sarmiento Garcia San Miguel** **Associate Professor Dr. Siti Suraiya Md Noor; MMC: 33300** **Dr. Rohimah Mohamud** **Dr. Tan Hern Tze** **Dr. Alwi Muhd Bestari @ Hashim; MMC: 43591**

INTRODUCTION

You are invited to take part voluntarily in research. This is about **the effects of nanoparticles from *mycobacterium smegmatis* on dendritic cells from human blood**

It is important that you read and understand this research information before agreeing to participate in this study. You will receive a copy from this form to keep. For your records if you agree to participate.

To identify the types of TB patients, some steps in the research are required but do not require you to take medicines (non-interventional research). It is important for you to read and understand the study information before you agree to participate in this research study. If you participate in this study, you will. Receive a copy of this form for your savings.

SCOPE OF THE RESEARCH

The scope of the study is focused on the patients who came for treatment at the HUSM Medical Specialist Clinic (KPP). Your participation in this study is expected tp take 15 minutes for 1 session for blood sampling by 10 ml by TB clinical physician of pulmonary TB by following the permitted procedure. A total of 46 TB patients are expected to participate in this study.

PURPOSE OF THE STUDY

This study is generally intended to investigate liposomes-based lipids from *Mycobacterium smegmatis* as a vaccine on white blood cells of patients with TB. This study is very important in expanding the potential of liposomes-Msmeg in medicine, especially TB disease.

General:

To investigate the effects of liposomes-Msmeg as a lipid-based vaccine on dendritic cells of human peripheral blood monocytes (PBMCs) of healthy and TB individuals

Specific:

1. To produce and characterize liposomes from total lipid of *M. smegmatis*
2. To determine the proportions of DCs in the PBMCs of healthy and TB patients
3. To determine the intracellular localization of liposomes-Msmeg by dendritic cells of healthy and TB individuals by using confocal microscopy and flow cytometry
4. To perform a correlation study between proportions of DCs in the PBMCs of sputum smear positive pulmonary TB
5. To perform a correlation study between phenotype (expression markers) and functional properties (uptake capacity) of sputum smear positive pulmonary TB

PARTICIPANTS CRITERIA

The research team members will discuss your eligibility to participate in this study. It is important that you are completely truthful with the staff including your health history [if relevant ONLY].

This study will include:

Inclusion criteria

TB patients:

- Age 18 to 60 years old
- Patients who had pulmonary TB with smear and / or culture positive to Mtb without treatment

Healthy individuals:

- The individuals without infection or symptom of TB

This study will not include individual:

Exclusion criteria

TB patients:

- Age below of 18 years old

- Use of immunosuppressive drugs
- HIV-Positive
- Pregnant women
- Breast feeding woman
- Use treatment for diabetic and hypertension

Healthy individuals:

- Below of 18 years old

If the participant is selected as a 'normal control' then it should state clearly on the inclusion and exclusion criteria using the similar template as above

STUDY PROCEDURES

Sampling method and subject requirement

- Patients diagnosed with pulmonary TB (with inclusion criteria) seeking treatment for the first time at KPP HUSM. Informed consent will be obtained voluntarily prior to sample collection

Data collection method

i. Pre-study preparation

- Investigator will be preparing a pre-packed bag consist of study related form, informed consent form and EDTA tube

ii. Patient recruitment

- Clinical physician of pulmonary TB at the KPP HUSM will identify the prospective patients/ subjects who matched to the inclusion criteria and consulted them. The corresponding patients / subjects involved are those who attend the clinic from February 2019 to December 2019. Once patient agree, informed consent and associated form will be issued and obtained. Honorium will be given to each consented patients / subject that involved. **No vulnerable issues involved in this study**

iii. Sample collection

- Blood samples (10 ml) will be collected by vein puncture after following the informed consent procedure by Clinical physician and signature of the corresponding form.

iv. Data collection

- Ethical permission for all the procedures involved in this study will be obtained from the Human Research and Ethical Committee (JePEM) in Universiti Sains Malaysia. With consent, patient's biodata will be taken from their records. Privacy and confidentiality will be of high importance. At the start of study enrolment, each participant will be given a unique study number

to identify them / the sample in the following process of the study, and names will not be revealed. Study forms and consent forms can only be handled by authorized study investigators and staff and kept in a locked cabinet within Department of Immunology, PPSP, USM. Any electronic database with participant names and information is protected and can be accessed only by study investigators.

RISKS

If you participate in this research, you may experience a pain when the needle is injected, bruised after the injection and a little blood will probably dropped out at the injection site. The effects are normal, and it will disappear within a short timeframe.

Please inform the staff who involved in this study if you encounter any problems or information that may change your agreement to continue participating in this study.

REPORTING HEALTH EXPERIENCES

Please contact, at any time, the following researcher if you experience any healthy problem either directly or indirectly related to the study.

Dr. Alwi Muhd Bestari @ Hashim (Number of Full Registration of Malaysian Medical Council: 43591) at 09-767 6572 or 012-4797577.

PARTICIPATION IN THE STUDY

Your participation in this study is entirely voluntary. You may refuse to take part in the study, or you may stop you participation in the study at any time, without any penalty or loss of benefits to which you are otherwise entitled. Your participation also may be stopped by the research team without your consent if in any form you have violated the study eligibility criteria. The research team member will be discussed with you if the matter arises.

POSSIBLE BENEFITS [Benefit to individual]

This study procedure will be provided to you at no cost. You may receive an information about your health from physical examination and laboratory tests to be performed in this study. The information and findings of this study are expected to benefit the patients in the future.

POSSIBLE BENEFITS [Benefit to community]

There is no negative impact on society. Blood donated by health individuals and Tb patients can help researchers to investigate the effects of liposomes-Msmeg as a lipid-based nanoparticles on humans to prevent Mtb infection. Therefore, liposomes-Msmeg can be used as vaccine for humans to prevent Mtb infection in turn to reduce the occurrence of TB disease.

POSSIBLE BENEFITS [Benefit to university]

There is no negative impact on the university. Results from this study may help researchers to develop potential TB vaccine, lipid-based nanoparticles. Thus, it will

help the university in the research and development of TB vaccine to encourage the collaboration between local and international researchers to develop more effective TB vaccine for the benefit of the world.

QUESTIONS

If you have any question about this study or your rights, please contact:

Dr. Alwi Muhd Bestari @ Hashim
Jabatan Perubatan, Pusat Pengajian Sains Perubatan,
Universiti Sains Malaysia, Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan
Office number: 09-767 6572

Profesor Madya Dr. Siti Suraiya Md Noor
Jabatan Mikrobiologi, Pusat Pengajian Sains Perubatan,
Universiti Sains Malaysia, Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan
Office number: 09-767 6247

Dr. Ramlah Kadir
Jabatan Imunologi, Pusat Pengajian Sains Perubatan,
Universiti Sains Malaysia, Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan
Office number: 09-767 6226

If you have any questions regarding the Ethical Approval or any issue / problem related to this study, please contact:

En. Mohd Bazlan Hafidz Mukrim
Setiausaha Jawatankuasa Etika Penyelidikan (Manusia) USM
Bahagian Penyelidikan dan Inovasi (P&I)
USM Kampus Kesihatan.
Office number: 09-767 2354 / 09-767 2362

CONFIDENTIALITY

Your information will be kept confidential by the researchers and will not be made publicly available unless disclosure is required by law.

Data obtained from this study that does not identify you individually will be published for knowledge purposes.

Your original records may be reviewed by the researcher, the Ethical Review Board for this study, and regulatory authorities for the purpose of verifying the study procedure and / or data. Your information may be held and processed on a computer. Only research team members are authorized to access your information.

By signing this consent form, you authorize the record review, information storage and date process described above.

MAINTENANCE OF SAMPLE STUDIES AND SUBJECTS PROCEDURES

The blood samples will be stored and processed in the culture laboratory of Immunology Department, PPSP, USM. Permission to access the research sample will only be permitted to the researchers and students who involved in this study only. Excessive blood samples (if any) will be stored in the -80^{o}C frozen refrigerator and then transferred to the fluid Nitrogen tank. This stored blood samples will be used in the next study procedure by your permission with the approval of HUSMs human ethical board. Otherwise, this stored blood sample with be discarded according to the prescribed procedure.

SIGNATURE

To be entered into the study, you or a legal representative must sign and data the signature page on Subject Information and Consent Form (**ATTACHMENT S**).

ATTACHMENT S

Subject Information and Consent Form
(Signature Page)

***Research title*: Deciphering the mechanisms of cellular uptake of natural liposomes derived from *Mycobacterium smegmatis* (liposomes-Msmeg) as a lipid-based vaccine by DCs of human peripheral blood monocytes (PBMCs) of healthy and patients with TB**

Researcher's Name: Dr. Ramlah Kadir

To become a part this study, you or your legal representative must sign this page. By signing this page, I am confirming the following:

- I have read all the information in this Patient Information and Consent Form **including any information regarding the risks in this study** and I have had time to think about it.
- All my questions have been answered to my satisfaction.
- I voluntarily agree to be part of this research study, to follow the study procedures, and to provide necessary information to the doctor, nurses, or other staff members, as requested.
- I may freely choose to stop being a part of this study at any time.
- I have received a copy of this Participant Information and Consent Form to keep for myself.

_____

Participant Name

_____

Participant I.C. No.

_____ _____

Signature of Participant or Legal Representative **Date** (dd/MM/yy)

_____

Name of Individual
Conducting Consent Discussion

_____ _____

Signature of Individual **Date** (dd/MM/yy)
Conducting Consent Discussion

_____ _____

Name & Signature of Witness **Date** (dd/MM/yy)

ATTACHMENT P

Participant's Material Publication Consent Form
(Signature Page)

***Research title*: Deciphering the mechanisms of cellular uptake of natural liposomes derived from *Mycobacterium smegmatis* (liposomes-Msmeg) as a lipid-based vaccine by DCs of human peripheral blood monocytes (PBMCs) of healthy and patients with TB**

Researcher's Name: Dr. Ramlah Kadir

To become a part this study, you or your legal representative must sign this page. By signing this page, I am confirming the following:

- I understood that my name will not appear on the material published and there has been efforts to make sure that the privacy of my name is kept confidential although the confidentiality is not completely guaranteed due to unexpected circumstances.
- I have read all the materials or general description of what the material contains and reviewed all photographs and figures in which I am included that could be published.
- I have been offered the opportunity to read the manuscript and to see all materials in which I am included but have waived my right to do so.
- All the published materials will be shared among the medical practitioners, scientists, and journalist worldwide.
- The materials will also be used in local publications, book publications, and accessed by many local and international doctors worldwide.
- I hereby agree and allow the materials to be used in other publications required by other publisher with these conditions:
- The materials will not be used as advertisement purposes nor as packaging materials
- The materials will not be used out of context – i.e.: Sample picture will not be used in an article which is unrelated subject to the picture.

_____

Participant Name

_____	_____	_____
Participant I.C. No.	**Participant's Signature**	**Date** (dd/MM/yy)

_____	_____
Name and Signature of Individual Conducting Consent Discussion	**Date** (dd/MM/yy)

Appendix A

Study form

Deciphering the mechanisms of cellular uptake of natural liposomes derived from *Mycobacterium smegmatis* (liposomes-Msmeg) as a lipid-based vaccine by DCs of human peripheral blood monocytes (PBMCs) of healthy and patients with TB

Specimen no: Date:

Study ID:

Mailing address:

Telephone no (Home/Mobile phone):

Age: Date of birth:

Gender: Race:

Clinical Data

History of tuberculosis: Yes ☐ No ☐

If yes,

Completed treatment ☐ Not completed treatment ☐ Reason: _____

Family history of tuberculosis Yes ☐ No ☐

If yes,

Parent ☐ Sibling ☐ Others: _____

Smoking: Yes ☐ No ☐

If yes, Active ☐ Passive ☐

Pulmonary TB with smear: Positive ☐ Negative ☐

Mtb culture: Positive ☐ Negative ☐

If positive,

With treatment ☐ Without treatment ☐

Use of immunosuppressive drugs: Yes ☐ No ☐

HIV-positive: Yes ☐ No ☐

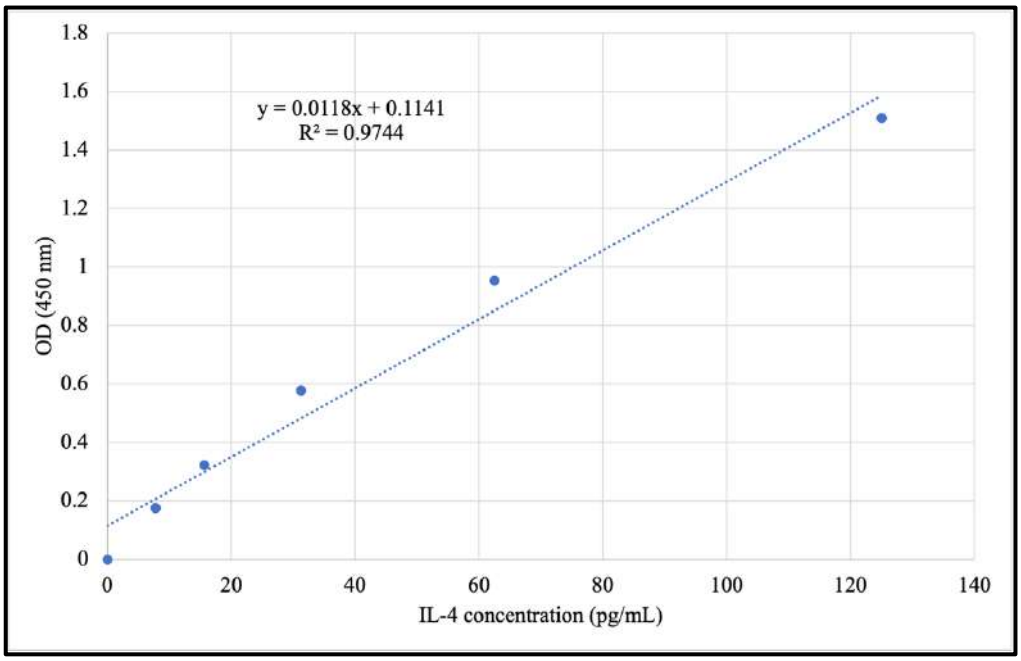
Pregnant women: Yes ☐ No ☐

Breast feeding: Yes ☐ No ☐

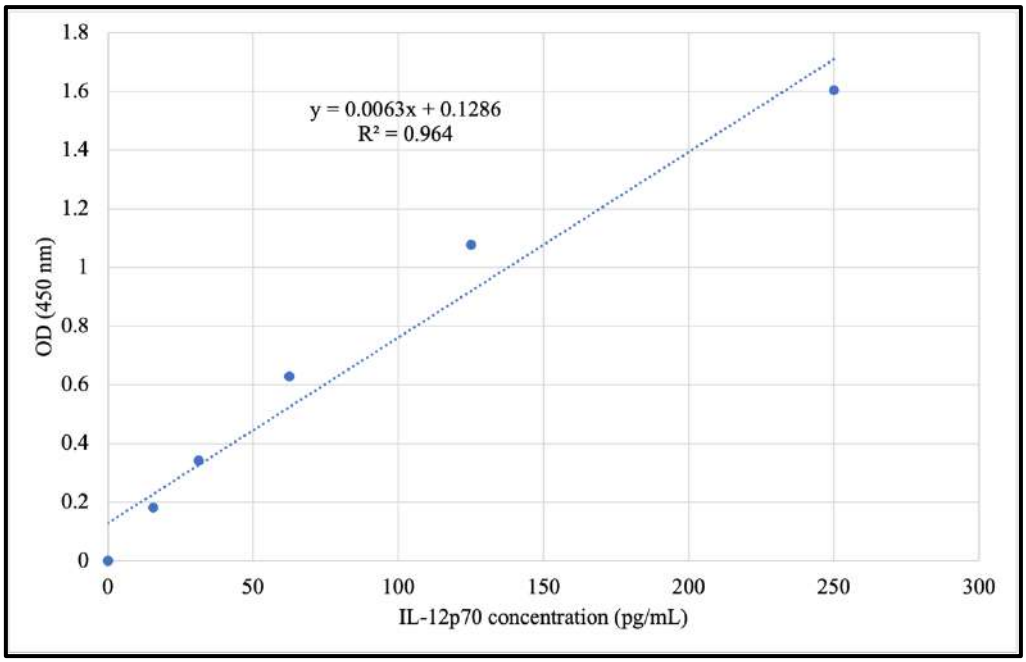
Use treatment for diabetic and hypertension Yes ☐ No ☐

Appendix C

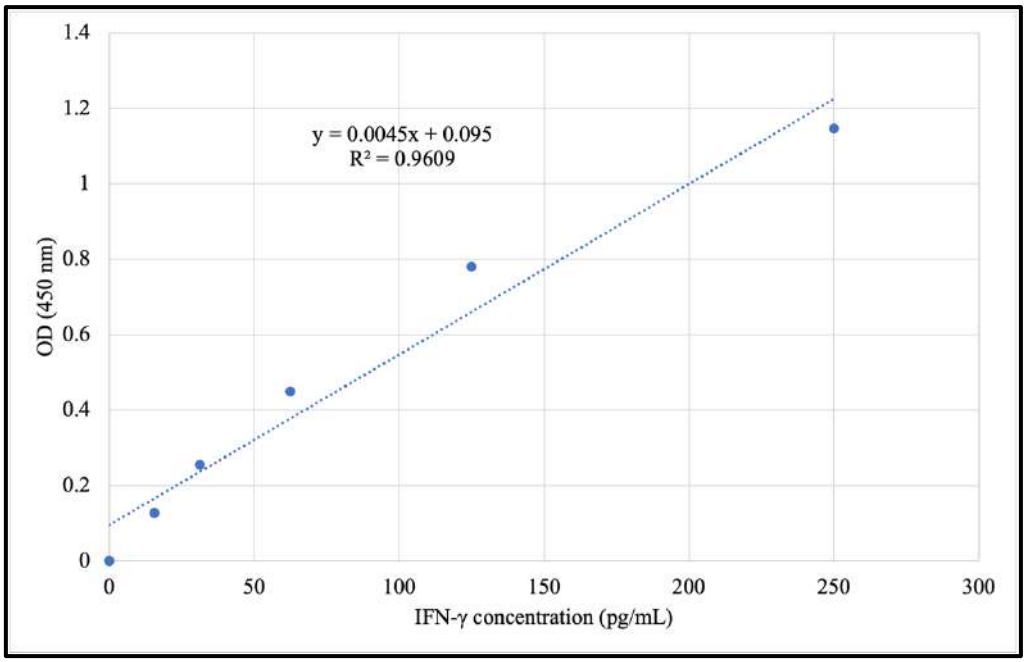
1. Standard curve IL-4



2. Standard curve IL-12p70



3. Standard curve IFN-γ



LIST OF PUBLICATIONS

Published Publications

1. Kadir, R., Ismail, N., Nasir, N. A. M., **Suhaimi, N. A. A.** (2020, December 23). *COVID-19: Calon vaksin yang telah memasuki fasa klinikal.* Universiti Sains Malaysia. http://covid19.kk.usm.my/artikel/bacaan-umum/95-covid-19-calon-vaksin-yang-telah-memasuki-fasa-klinikal

2. **Suhaimi, N. A. A**., Ahmad, S., Husna, S. M. N., Sarmiento, M. E., Acosta, A., Norazmi, M. N., ... & Kadir, R. (2022). Application of liposomes in the treatment of infectious diseases. *Life Sciences*, *305*. **(JCR IF: 6.78, Q1)**

3. Luwi, N. E. M., **Ahmad, S., Azlyna, A. S. N**., Nordin, A., Sarmiento, M. E., Acosta, A., ... & Kadir, R. (2022). Liposomes as immunological adjuvants and delivery systems in the development of tuberculosis vaccine: A review. *Asian Pacific Journal of Tropical Medicine*, *15*(1), 7. **(JCR IF: 1.226, Q3)**

4. Lambuk, L., **Suhaimi, N. A. A**., Sadikan, M. Z., Jafri, A. J. A., Ahmad, S., Nasir, N. A. A., ... & Mohamud, R. (2022). Nanoparticles for the treatment of glaucoma-associated neuroinflammation. *Eye and Vision*, *9*(1), 1-29. **(JCR IF: 4.427, Q1)**

5. Hatmal, M. M. M., Al-Hatamleh, M. A., Olaimat, A. N., Ahmad, S., Hasan, H., **Ahmad Suhaimi, N. A**., ... & Mohamud, R. (2022). Comprehensive Literature Review of Monkeypox. *Emerging Microbes & Infections*, 1-84. **(JCR IF: 19.568, Q1)**

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1. Kadir, R., Mohamud, R., Yusop, N., Zulkafli. N. E. S., **Suhaimi, N. A. A.**, Nordin, N. A. *Tuberkulosis penyakit tibi pulmonari dan ekstrapulmonari tibi*. Copyright 2023

2. Kadir, R., Mohamud, R., Yusop, N., Zulkafli. N. E. S., **Suhaimi, N. A. A.,** Nordin, N. A. *Tuberkulosis diagnosis makmal.* Copyright 2023

3. Kadir, R., Mohamud, R., Yusop, N., Zulkafli. N. E. S., **Suhaimi, N. A. A.,** Nordin, N. A. *Tuberkulosis.* Copyright 2023

4. Kadir, R., Mohamud, R., Yusop, N., Zulkafli. N. E. S., **Suhaimi, N. A. A.,** Nordin, N. A. *Tuberkulosis tibi dan jangkitan bukan tibi*. Copyright 2023

Oral presentation

1. '*In vitro* uptake and activation of human dendritic cells by liposomes derived from total lipid from *Mycobacterium smegmatis*' was presented as an oral presentation at the 6th International Conference on Molecular Diagnostics & Biomarker Discovery (11-13th October 2022, WEBEX online)

Life Sciences 305 (2022) 120734

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Review article

Application of liposomes in the treatment of infectious diseases

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ARTICLE INFO

Keywords:
Drug delivery system
Nano-carrier
HIV
Liposomes
Malaria
Tuberculosis
Vaccines

ABSTRACT

The advances in the development of drugs and vaccines for major infectious diseases of tuberculosis (TB), malaria and HIV represent some of the most significant milestones in their therapeutic strategies. Yet, current drugs and vaccines display limitations such as drug resistance and low efficacy level. In recent years, new emerging and advanced nano-technology carrier liposomes have been widely studied towards producing drugs and vaccines capable of targeting infectious diseases. Liposomes portrayed biocompatible and biodegradable properties with versatile flexibility, characteristics that are advantageous for a good targeting at the site of action. The success of liposomes has renewed interest in the research and development of liposomal drugs and vaccines shifting the paradigm in infectious diseases treatment. This review focuses on the limitations of current therapeutic drugs and vaccines, the knowledge of liposomes in terms of their classifications and advantages, and a review of the application of liposomes in the treatment of TB, malaria, and HIV infection.

1. Introduction

Infectious diseases are a global health concern. Infectious diseases are caused by various pathogenic microorganisms: bacteria, fungi, parasites, and viruses which can be transmitted between people through direct bodily contact, air droplets, vehicle spreads, zoonoses, and vector-borne transmission [1]. Malaria, tuberculosis (TB), and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) are the top three major infectious diseases worldwide [2]. The highest mortality rate is seen in low-income countries with a mortality rate of 4 million per year [2,3]. The gold standard in the treatment of these diseases is the use of vaccines and antibiotics. Vaccination is crucial in disease prevention and had successfully eradicated smallpox and controlled several other infectious diseases [4]. However, vaccines against malaria and HIV are not commercially available, although there are several potential candidates that had entered clinical trials [5,6].

Meanwhile, the TB vaccine which solely relied on neonatal BCG vaccination is highly known for its inefficiency to reduce *Mycobacterium tuberculosis* (Mtb) transmission among adolescents and adults [7–9]. Despite the great efficacy shown by antibiotics in these disease treatments [10], there are limitations in the current antibiotic regime including poor adherence to medicine among patients, poor tolerability, limited access to medications due to high costs and resistance, the emergence of major side effects and low efficacy of antibiotics [11–14].

Thus, it has been proposed that liposomes can overcome the limitation of currently available drugs and vaccines. Liposomes are nanotechnology-based carrier in drug delivery carrier. In drug delivery, liposomes are spherical artificial vesicles, surrounded by a membranous lipid bilayer of phospholipids [15]. Both natural and synthetics derivatives can be used as the components of phospholipids in a liposome's preparation. Primarily, liposomes are used as drug delivery systems (DDS) and adjuvant and/or antigen carriers in vaccines. Early pioneers of liposomes, Gregoriadis and Perrie, described the liposome's ability to encapsulate drugs and efficiently deliver them to the target sites [16]. This narrative review focuses on the limitations to current therapeutic drugs and vaccines that persist in the treatment of infectious diseases, the basic knowledge of liposomes such as classification of liposomes, the advantages of liposomes, and reviews the use of liposomes in the treatment of TB, malaria, and HIV.

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https://doi.org/10.1016/j.lfs.2022.120734
Received 5 February 2022; Received in revised form 8 June 2022; Accepted 22 June 2022
Available online 24 June 2022
0024-3205/© 2022 Published by Elsevier Inc.

Table 1
Limitations of therapeutic drugs and vaccines in infectious diseases.

Limitations	References
Drugs	
Antibiotic resistance	[19]
High toxicity level	[21]
Poor bioavailability	[27]
Short plasma life	[26]
Rapid drug elimination from the body	[26]
Serious adverse effects	[31]
Distribution of poor-quality drugs	[32]
Vaccines	
Potential side effects	[34,43]
Variable temperature-controlled storage	[36]
Age-dependent suitability	[40]
Responses - humoral immunity but not cellular immunity	[34]
Costly	[37]
Repeated doses required	[42]
Human error	[42]

2. Limitations of current treatments in infectious diseases

2.1. Limitation of current therapeutic drugs

Despite the advancement in rapid identification of causative agents in these diseases being helpful in managing and reducing the transmission of these diseases over the centuries [17,18], there have been several limitations of the therapeutic drugs. The lack of highly effective and efficient drugs and the emergence of drug resistance (third-line antibiotics) remain major limitations in the current management of infectious diseases [19]. It is reported that multidrug-resistant (MDR) TB widely affect patients in China, Russian Federation, and India [20]. Another drawback of the current antibiotic regimes is the induction of toxicity [21]. Although additional supplementation of drug dosage can be beneficial, typically for treating severe infections, it is shown to induce drug toxicity and reduce therapy compliance which in turn contribute to the occurrence of antibiotic resistance [22,23].

Another limitation of current drug therapy of infectious diseases is the reduced drug bioavailability at the targeted site [24]. Routes of administration and dosage are the main factors to determine the drug's bioavailability rate and extension [25]. Most drugs in the market are commonly administered through oral or parenteral routes, which resulted in several first-pass effects such as drug degradation and low absorption as well as rapid clearance from the body [26,27]. Due to protein pharmacokinetic properties, several factors such as molecular weight, size, and surface charges determine the elimination rate [28–30]. Thus, a higher dose of the drug is usually needed to maintain a therapeutic concentration during the desired period [28]. This would cause an after-effect due to the fluctuation of the therapeutic with a high initial peak. It is proposed that the extension of plasma half-life in future biologicals can improve the efficacy of drugs, even with lower doses.

Failure to adhere to prescribed regimens, mostly due to the prescription of multiple drugs in disease treatment, serves as a limitation. Multiple drug intervention is associated with high costs and serious adverse effects [31]. Moreover, the distribution of low-quality drugs to diverse countries and provinces, as well as the low socio-economic status of affected populations are global concerns in eradicating infectious diseases [32]. These challenges need an urgent solution and liposomes are proposed as a highly potent drug carrier [33]. A summary of the limitations of therapeutic drugs is presented in Table 1.

2.2. Limitations of current vaccines

Vaccination is a well-established and effective method in infectious disease management. However, conventional vaccines which are commonly prepared from attenuated or killed microorganisms or part of their fragments to induce immunity possessed several limitations. This method is often linked with undesirable side effects such as allergic responses due to ingredients (*i.e.* active substance in residuals from preparation) in the vaccines [34]. The cold chain is important to preserve vaccine potency therefore mass production and use of such vaccines is not always achievable and affordable [35,36].

New generation vaccines known as subunit vaccines (*i.e.*, composed of carbohydrates, recombinant DNA, purified proteins, or peptides) are less or non-immunogenic require the addition of adjuvants. The safety of adjuvants in immunocompromised and elderly patients still needs to be elucidated [36]. Besides, antibodies created from the induction of immunity are restricted to humoral immunity only, usually without or only weak cellular immunity being produced [34]. Efficient vaccines should significantly enhance both cellular and humoral immune responses and this can be achieved by using an appropriate particle-based vaccine delivery system such as liposomes.

High production cost (~100 million USD) from concept to market and long development process (*i.e.*, takes 5 to 18 years) still great limitations in vaccine production [37,38]. Flexible, rapid, and low-cost vaccine development and manufacturing technologies are required to meet the high demands of vaccine supply, especially highly-populated regions [39]. Vaccines that require multiple doses for optimal immunogenicity and efficacy also means a time-consuming process [40,41]. Thus, close monitoring must be conducted to evaluate compliance with vaccination schedules, especially in infants and young children. New innovative vaccines aim to reduce doses and achieve the same efficacy and optimal disease protection in the population.

Parenteral vaccination is the gold standard in vaccine immunization, however, it requires expert practitioners to handle it and there is a needles and syringes issue by way of needle-phobia or accidents with used needles [42]. Alternatively, the administration of oral vaccine which are currently limited can be implemented. However, the oral vaccine can cause a harsh gastrointestinal environment and tolerance induction and require a higher dose of antigen compared to parenteral immunizations, and this requires a solution to cater to the issues [43–45]. To overcome these limitations, liposomes are used to increase immunogenicity without additional side effects.

3. Liposomes

Liposomes have amphiphilic characteristics, and are composed of a hydrophilic head and hydrophobic chains that interact with the aqueous phase, suggesting a close resemblance to the cell membrane [47–49]. Hydrogen bonds and polar interactions between the lipophilic permeable membrane and polar heads of lipids contribute to the stabilization of liposomes [50]. The lipid molecular structure that possesses both water-hating and water-friendly moieties enables liposomes to entrap and solubilize numerous insoluble and water-soluble drugs into the bilayer and aqueous core of the liposomes respectively [51–55]. Phosphatidylcholine (PC) and dipalmitoyl PC in an aqueous medium are the most common human lipids used in liposome preparation and these substances can induce potent immune responses [56,57]. Owing to their unique versatile structure, a variety of molecules (*e.g.*, proteins, peptides, and nucleic acids, lipids) can be incorporated into liposomes [58,59].

The size of liposomes ranging from small vesicles (0.025 μm) to larger vesicles (2.5 μm) influences the encapsulation of drugs and their half-life [47,53]. Flexible characteristics of liposomes include the ability of surface modification that can prolong the circulation time of drugs and vaccines [60]. A hydrophilic polymer such as polyethylene glycol (PEG) shows higher repulsive forces between liposomes and serum components, with the enhancement of permeability and retention (EPR) effects and helps to prolong their half-life as well as delivery of biomolecules to the site of action [61–63]. The size and stability of liposomes are crucial to be maintained to avoid drug leakage that will reduce the efficacy of drugs. A proper storage condition in a dry state

Table 2
Classification of liposomes.

Criteria to differentiate liposomes	References
Structural parameters	[89]
• Unilamellar vesicles • Multilamellar vesicles • Oligolamellar vesicles • Multivesicular vesicles	
Composition	[90]
• Conventional liposomes • Cationic liposomes • Long circulatory (stealth liposomes) • Fusogenic liposomes • pH-sensitive liposomes • Immuno-liposomes (tagged by antibodies or ligands)	
Methods of preparation	[92]
• Active loading technique • Passive loading technique	

could make the liposomes last up to three months upon incubation at 4 °C, with the addition of stabilizers (*e.g.*, sucrose) by minimizing free drug concentration and reducing the alteration in vesicle size [64–66]. The use of appropriate excipients within the liposomal formulation protects the liposomes during the freeze-drying process [67]. The most used excipients in freeze-drying of pharmaceutical products include bulking agents (*e.g.*, trehalose, mannitol, lactose), buffer stabilizers (*e.g.*, glycine, alanine, lactose), tonicity adjusters (*e.g.* mannitol, glycine, glycerol) and collapse temperature modifiers (*e.g.* hydroxypropyl-β-cyclodextrin, PEG and dextran) [68].

The diversity of microorganisms such as bacteria, protozoa, fungus, and viruses has been associated with severe human infections [69]. Current therapeutic drugs have limitations that become a major drawback in disease management. Therefore, liposomes produce a potent efficacy, overcoming the limitations such as in fungal disease by improving the local effects of the drug through a deeper penetration to the specific site with sufficient drug distribution and good recovery of organs [70–72]. Previous findings also demonstrated liposomal encapsulation significantly increased the survival rate of fungal-infected mice with reduced fungal burden in lung tissues [73–75]. Moreover, in a protozoan study, the incorporation of liposomes even promotes a significant therapeutic index, which could lower the toxicity and regulate the release of drug to the target cell site, where the protozoa parasites reside [76]. Certain drugs, especially those against bacteria, have low pharmacodynamic activities that require a higher dosage concentration which can induce other side effects. Addressing this challenge, drug-encapsulated liposomes studies demonstrated a greater immunogenicity effect which rejuvenates the immune cells and antibody secretion with rather reduced complication [77]. These findings provide an insight into the depletion of inflammation markers and longer circulation time by liposomes to adjust their physicochemical properties and stimulate highly efficient outcomes in infectious diseases management [78,79]. Furthermore, liposomes are competent as immunoadjuvant which elicits strong humoral immune responses *via in vitro* $CD4^+$ and $CD8^+$ T cells [80]. This property allows liposomes to enhance the stimulation of cytokines (such as IFN-γ, IL-4, and IL-2) and HIV-specific antibody production [81,82]. Collectively, these findings provide a wide research platform to exploit liposomes due to its extraordinary capabilities and good safety aspects.

The mechanisms involved in the interaction of liposomes and cells during drug delivery may include liposomal adsorption to the surface of the cell membrane followed by the process of releasing the drug. The lipid bilayer of the liposome diffuses with the lipoidal cell membrane which allows direct transmission into the cytoplasm and results in the exchange of lipid [16,83]. In addition, liposomes also function as effective carriers for adjuvants and mediators due to their unique properties which can be adjusted during the formulation process, through the composition of immune-stimulators, incorporation of lipids and antigen immune-stimulators [84,85]. Taken together, liposome is a promising nanocarrier application in drug and vaccine intervention in infectious disease treatment.

Table 3
Advantages of liposomes as drug delivery systems and tools in vaccine development.

Application	Properties	References
Drug delivery system	Amphiphilic structures to trap hydrophobic, hydrophilic drugs	[49]
	Good targeting availability by active and passive targeting	[100]
	Biocompatibility suggests minimum toxicity levels	[112]
	Biodegradability prevents physiological degradation	[113]
	Membrane fluidity	[108]
	Versatile structure	[59]
Vaccine	Potent immune response inducer	[56]
	Versatile structure in terms of size, lamellarity, surface modification	[53]

3.1. Classification of liposomes

The nature of a liposome nano-system is governed by some structural parameters (type of vesicles, sizes, and several lipid bilayers), composition, and method of preparation (Table 2) [47,86–88]. Liposomes are divided into four major groups: i) unilamellar vesicles (ULV), ii) multilamellar vesicles (MLV), iii) oligolamellar vesicles (OLV), and iv) multivesicular vesicles (MVV). The various sizes of ULV include small, medium, large and giant vesicles [89]. A diverse array of liposome compositions has been developed including conventional liposomes, cationic liposomes, long circulatory (stealth) liposomes coated with polymeric conjugated, fusogenic liposomes, and pH-sensitive liposomes and immuno-liposomes (direct tagging by antibody or ligands) [90,91]. Active and passive methods are the main methods in the preparation of liposomes [92,93]. Proliposomes lyophilization is an example of an active preparation method. It is beneficial in increasing the ice sublimation rate upon lipid dissolution into tert-butyl alcohol (TBA)/water solution to prevent materials from collapsing and is highly known to be a cost-effective technique [94]. In passive loading, methods used are mechanical dispersion, solvent dispersion, and detergent removal [47].

3.2. Advantages of liposomes

Liposomes have been studied in numerous diseases such as cancer, autoimmune diseases, and allergy through two different strategies: passive and active targeting [95–97]. Nevertheless, liposomes encounters some a few challenges such as a high production cost, low solubility rate, reduced shelf-life, chemical instability that can affect the encapsulation efficiency, sterilization towards microbial contamination, and lenkage of the encapsulated drugs (Table 3) [98]. For instance, although cationic liposomes possess a relatively weak adjuvant activity and instability, studies showed better results in the combination with other immunostimulatory agents such as trehalose dimycolate, trehalose 6,6′dibenehate and monophosphoryl lipid A [99]. Moreover, passive targeting by liposomes (*i.e.*, involves surface coating with polymer) can prevent the uptake of drugs by the reticuloendothelial system (RES), allowing prolonged circulation of drug-loaded liposomes. Active targeting (*i.e.*, through ligand-receptor-specific binding) additionally improves specific accumulation to the site of action and minimizes off-target effects [100–102]. Soluble rifampicin incorporated into liposomes resulted in rapid delivery and prolonged release of the drugs to macrophages with minimized systemic side effects compared to free rifampicin drugs [103].

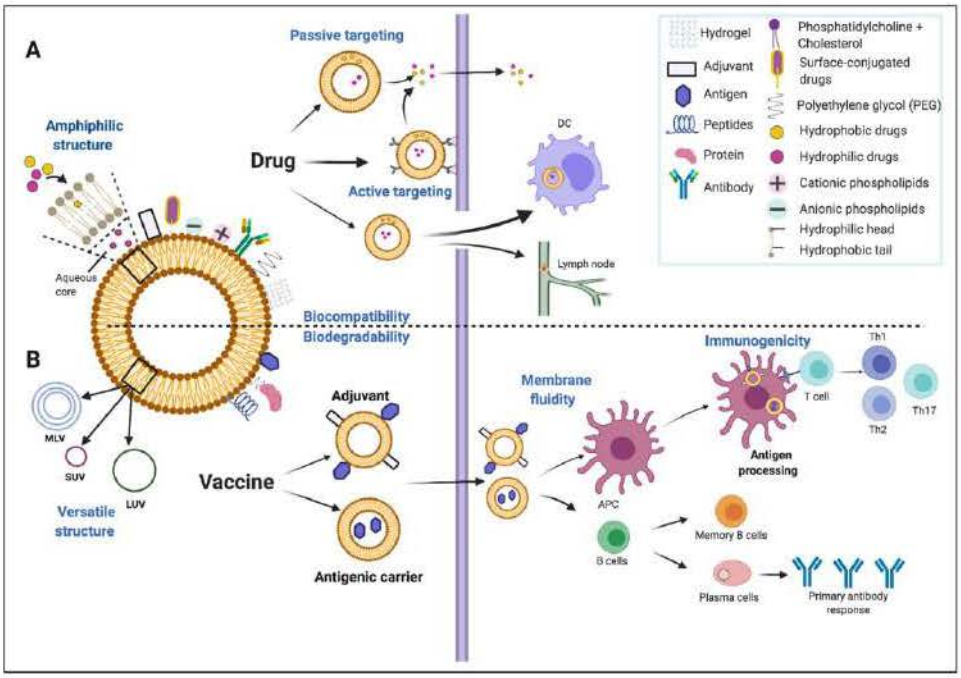

Fig. 1. Unique properties of liposome and its application mechanisms in drugs and vaccines. Liposomes benefit from their unique properties such as amphiphilic structure, their versatile structure in terms of size, lamellarity, surface modification, and good targeting availability by active and passive targeting, plus, their biocompatibility and biodegradability properties.

The advantage of liposomes is it has biocompatibility and biodegradability features (Table 3). Significant biocompatibility of liposomes is shown by high reduction of cytotoxicity with a minimum level of toxicity [104,105]. Biodegradability (*i.e.* the ability of the compounds to be cleared naturally from the body) features of liposomes is due to the biodegradable materials (*e.g.* chitosan, gelatine, dextran, and alginate) used to make liposomes. These decompose easily and reduce adverse effects to the host [106,107]. Lipids such as PC and dipalmitoyl PC used in liposomes help to induce potent immune responses, a very encouraging finding in the potential of liposomes use in vaccine development [56,57]. As in liposomal drug delivery systems, the concentric lipid bilayers of liposomes as adjuvant and antigenic carriers in vaccines are designed using phospholipids.

Length chain, saturation degree, and location of hydrocarbon (*i.e.* influences on membrane fluidity) are considered in creating the bilayer components [108]. A rigid structural bilayer is formed by a longer hydrogen chain, while, a shorter tail results in fluid bilayer formation. Liposome adjuvants enhance the recognition of cellular uptake, antigen processing, and presentation by antigen-presenting cells (APC) and antigen-specific immune responses (both humoral and cellular) [106,107,109–111]

Taken together, liposomes are promising nanocarriers in delivering drugs and vaccines. Unique properties of liposomes as a highly potential DDS and in vaccine development are illustrated in Fig. 1.

4. Liposomes and tuberculosis

4.1. Liposomes for tuberculosis treatment

Tuberculosis (TB) is the leading cause of death globally with approximately 1.45 million deaths in 2018 [114]. An estimated 1.7 million people are infected with Mtb, the mycobacteria that cause TB [115]. Thus, developing intervention against TB represents a crucial global health priority. Primarily, TB pathogenesis involves invasion of Mtb into the lungs and uptake by alveolar macrophages, thus initiating infection responses [116]. After macrophage phagocytosis, Mtb defends itself from macrophage killing and replication *via* several mechanisms [117]. The macrophage invades underlying epithelium and monocytes from nearby blood vessels and forms granulomas. The intracellular macrophage signalling changes the cytokine environment, eventually modifying the protective immune response (*i.e.*, leading to Mtb tolerance by the host and intracellular survival of Mtb over time) as well as initiating both innate and adaptive immune responses [118–120].

A latent TB infection exists, in which a granuloma represents the state of containment of the Mtb infection. Reactivation of latent TB (active TB) occurs upon the disruption of granuloma which then leads to the transmission of the infection. A bigger concern for global health security is the drug-resistant of TB. The primary resistance is towards the most effective first-line drug rifampin (RIF) and multidrug-resistant TB towards RIF and isoniazid (INH) [121–123]. The physicochemical properties of liposomes offer great potential in the management and

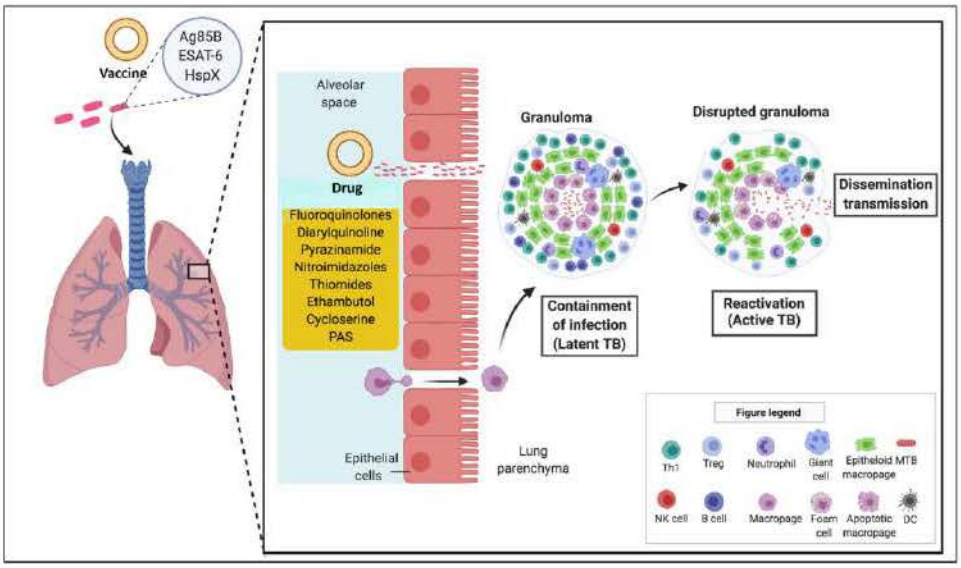

Fig. 2. Liposomes in TB as DDS and vaccine development. Liposomes encapsulated drugs that inhibit mycobacterial proliferation, meanwhile, in vaccine development, liposomes function to carry vaccine candidates such as Ag85B, ESAT-6, and HspX, and as adjuvants that enhance the effectiveness of the vaccine.

prevention of TB, both as DDS and as components in TB vaccines. Specifically, these liposomes are efficient in targeting macrophages, the specific host of TB. There are a few applications based on liposomes in the treatment of TB and the next section focuses on the encapsulation of drugs targeting Mtb and the adjuvant effects of liposomes for effective TB treatment.

4.2. Liposomal drug encapsulation products for TB

Encapsulation of multiple drug therapies within liposomes in pulmonary TB treatment is shown to have a greater entrapment efficiency, effective, sustained and prolonged drug release, plus enhanced recovery, and uptake of drugs from the affected or target site *in vitro* and *in vivo* [124]. Liposomes-encapsulate TB drugs such as RIF, INH, and pyrazinamide, could be further attached to another targeted receptor such as mannan, a ligand for drug delivery to the receptor site, providing maximum delivery and internalization [125]. In addition, the liposomal formula showed maximum drug release *in vitro* (simulated lung fluid and interstitial fluid) with enhanced relative bioavailability compared with the free unencapsulated drugs. Increased bioavailability of INH encapsulated in PC and cholesterol-containing 4-(5-pentadecyl-1,3,4-oxadiazol-2-yl) pyridine) liposomes showed up to 90 % reduction of live bacilli in the lungs of a drug-sensitive or MDR-TB animal models [125]. A study on RIF-loaded dry powder/freeze-dried liposomes (i.e. optimized formula of lecithin to cholesterol ratio of 60:40) showed a greater *in vitro* anti-tubercular activity against Mtb H37RV strain than the pure RIF alone [103]. A novel liposome-in-hydrogel system using biodegradable polymers with a derivative of INH (*N*′-dodecanoylisonicotinohydrazide) for *in vivo* localized treatment of bone TB demonstrated a rapid release of the drug into the synovial fluid to reach effective inhibitory concentrations after localized injection while maintaining a sustained drug release for several days [126]. This study shows a promising window of opportunity in the development of bone TB therapy.

A comparison study was done on the original liposomes/hydrogel assembly by creating drug-loaded liposomes entrapped within a chitosan (CS) physical hydrogel, a "drug-in-liposomes-in-hydrogel" (DLH), alongside a "drug-in-hydrogel" (DH) system [127]. RIF was loaded into 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine liposomes, and the cumulative release profiles of RIF were found to be lower from the DLH assembly in comparison to DH systems. Liposomes made of lecithin (CL) were used to co-encapsulate RIF and INH for liposomal dual delivery and demonstrated 90 % and 59 % encapsulation efficiency for RIF and INH, respectively [128]. Besides, liposomes containing lecithin exhibited a controlled release profile for co-encapsulated drugs [128]. These observations demonstrate liposomes' capability to encapsulate drugs efficiently, providing a delayed-release property for the drug. In another independent study, the pH-dependent release behaviour of isonicotinic acid (4-hydroxy-benzylidene)-hydrazide (INH-HB) encapsulated in CL liposomes showed a rapid release of INH in acidic media of up to 100 % release compared to basic media with only 22 % of INH released [129]. These INH-HB-loaded liposomes seem to be an attractive pH-responsive system considering the intra-macrophage site of the mycobacterium and pH-dependent phagocytic processes involved.

Cardiolipin on Mtb is shown to be a potential target for resistant bacteria and there exists a cardiolipin-based liposomal form of levofloxacin (LF) [130]. Liposomes consisting of cardiolipin (concentration of 335 μM) alone completely suppressed the growth of Mtb and incorporation of LF to phosphatidylcholine/cholesterol/cardiolipin further reduces the minimum inhibitory concentration of cardiolipin (*i.e.*, reduced concentration to 33.5 μM). Besides, the increase of antituberculosis activity of LF was directly dependent on the content of cardiolipin in the liposomes, suggesting the greater inhibitory effect of cardiolipin in liposomes on Mtb viability. This is further evidence of how the composition and ratio of lipid in liposomes affects the encapsulation efficiency, hence the efficacy of the drugs towards targeted sites. Overall, the observations represent good enforcement in the use of

liposomes as a drug carrier in multiple drug therapy.

4.3. Liposomal-based adjuvant products for TB vaccines

Cationic liposomes could be applied as adjuvants for the enhancement of antigenic peptides that increase the potential of the TB subunit vaccine [99]. Several adjuvant strategies and formulations using liposomes have been studied to improve the limitation of the current TB vaccine. A study investigating the effects of BCG vaccine using Mtb fusion protein encapsulated in liposomes comprising dimethyldioctadecylammonium/trehalose-6,6′-dibehenate (DDA/TDB), showed a greater Th1 response *in vivo* (IFN-γ, IL-12) than the BCG alone, indicating that liposomes possessed its own immunogenicity, enabling it to further amplify the protection of BCG [131].

The same efficacy using DDA/TDB liposomes for the BCG vaccine for TB was reported in other studies [132–134]. Recently, a novel liposome-based subunit vaccine formulation (Lipo-AE) based on phosphatidylserine, encapsulating two prominent TB antigens (Ag85B and ESAT-6) was developed [135]. The Lipo-AE formulation combined with PolyIC adjuvant was observed to boost the accumulation of resident memory T cells in the lungs after the immunization regimen based on *in vitro* systemic delivery [135].

More interestingly, the Lipo-AE vaccine candidate used on BCG-immunized mice, subsequently challenged with a low dose of aerosol Mtb *in vivo* showed a significant depletion of bacterial load in the lungs and spleen compared to immunization with BCG alone. Another liposome-based adjuvant containing de-O-acylated lipooligosaccharide (dLOS) aimed to improve immunogenicity and protective efficacy of TB subunit vaccines [136]. It was shown that the dLOS adjuvant formulation significantly amplified both humoral and Th1-type cellular responses to the TB subunit vaccine (*i.e.*, Ag85A, ESAT-6, and HspX). In addition, this liposomal adjuvant also proved to be a potential booster as portrayed by effective induction of Th1-type response in the BCG-primed mouse model. Lastly, protective efficacy was demonstrated by the dLOS/dimethyl dioctadecyl ammonium bromide-adjuvanted TB vaccine towards Mtb infection *in vitro* and *in vivo*. This suggests that this liposomal adjuvanted TB vaccine would be a promising vaccine candidate for the establishment of a booster vaccine.

Thus, liposomes' advantageous physicochemical properties provide a great tool in the development of a new therapeutic approach for TB as it is pivotal in substantively managing the expenditure of treatment, reducing interactions with anti-HIV drugs, and improving MDR-TB and latent TB. A summary of liposomal drugs and vaccines as well as their mechanisms in TB is presented in Fig. 2.

5. Liposomes and malaria

5.1. Liposomes for malaria treatment

Malaria is one of the most common vector-borne diseases, caused by the *Plasmodium* parasite of infected female Anopheles mosquitoes bites (malaria vectors) [137]. World Health Organization (WHO) reported approximately 229 million malaria cases in 2019 affecting over 87 malaria-endemic countries [138]. The pathogenesis of malaria infection occurs by the injection of sporozoites which travel from the salivary glands of the mosquito through the bloodstream of a human host to the liver, where they invade hepatocytes and multiply asexually. Thousands of daughter merozoites are released into the bloodstream by matured schizonts, invading the red blood cells. In certain infected red blood cells, the merozoites differentiate into sexual erythrocytic stages (gametocytes), which circulate in the bloodstream and are ingested during mosquito bites. The ingested gametocytes develop into mature sex cells (gametes) and elongated embryonic forms (ookinetes) that invade through the mid-gut wall of the mosquito and form oocysts, which release thousands of active sporozoites into the salivary glands of the mosquito. The cycle of human infection will repeat through the next mosquito bite [139]. Travassos et al. elucidated the erythrocytic phase, where malaria symptoms are most likely to be visible, as the level of infection that can be targeted with antimalarial drugs [140].

There are several classes of ongoing antimalarial drugs such as primaquine (PQ), chloroquine (CQ), and artemisinin that possess adverse side effects [141]. To overcome this limitation, antimalarial drugs encapsulated into liposomes have been showed to have better efficacy. This is discussed in the next section.

5.2. Liposomes incorporated with antimalarial drugs

Chloroquine (CQ), the primary antimalarial drug, inhibits the synthesis of parasitic protein by polymerizing toxic heme that was released during hemoglobin proteolysis in plasmodium vacuoles [142]. The emergence of CQ resistance has led to the identification of molecular markers; *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) and *Plasmodium falciparum* multidrug resistance protein-1 (*pfmdr1*) mutated gene, which can be targeted using liposomes [143,144]. Liposome modification with polyethylene glycol (PEG) is a potent approach to prolong the half-life of CQ drugs and enhance the target efficacy [145]. A formulated CQ-PEG liposome demonstrated higher apoptotic blood parasites in comparison to single-CQ drugs, with an improved clearance of parasites within the infected mice and delayed the endpoint [146]. In a different study, platinum-CQ diphosphate dichloride (PtCQ)-PEGylated liposomes promoted a minimum leakage of PtCQ drug [147]. Primaquine (PQ) is a potent drug partner commonly used with other antimalarial chemoprophylactics, especially CQ. PQ can kill gametocytes and prevent relapses of *Plasmodium ovale* and *vivax* in malaria [148–152]. However, because PQ possesses severe toxicity and adverse side effects, PQ incorporation with liposomes is highly recommended [153]. Single intravenous injection of natural phospholipid-based liposomes containing PQ exhibited competent anti-malarial activity with low toxicity, which led to full recovery in treated mice [154,155]. Higher bioavailability of PQ encapsulated liposomes in targeted organs with a moderate elimination when compared to the free PQ drug was demonstrated [156]. Another experiment that combined PQ and CQ into single liposomes showed reduced toxicity with efficient encapsulation in dual drug delivery of natural phospholipid-based liposomes [148,157–159]. These studies eventually highlighted the effect of liposomes to be used as a potential malaria therapy.

Another class of prominent antimalarial drugs is artemisinin and its derivatives; isolated from the sweet wormwood *Artemisia annua* [160]. Artemisinin is effective in all stages (erythrocytes, recrudescence, and gametocytes) of the malaria life cycle by inhibiting the growth of *Plasmodium* spp. [161]. In general, antiparasitic heme-iron activates artemisinin *via* endoperoxide ring breakdown, before releasing carbon-centered free radicals which kills the malarial parasites [160,162–165].

Drug resistance and short half-life are common limitations of artemisinin [166]. Artemisinin incorporated into PEGylated liposomes has been shown to produce a rapid absorption with longer bioavailability [167]. This finding was supported by a subsequent study artemisinin-PEGylated liposomes that showed a significant pharmacological effect compared to other controls [167]. In addition, artemisinin in synthetic liposomes also demonstrated rather similar results with higher artemisinin encapsulation and rapid release of artemisinin [32,168]. These studies demonstrated that artemisinin encapsulated by liposomes demonstrated a greater therapeutic effect compared to free artemisinin against malaria.

5.3. Liposomal vaccines for malaria

The complex life cycle of *Plasmodium*, in terms of its biological structure, various development stages, and cellular immune responses has remained the major challenge in the production of malaria vaccines [169,170]. Unlike other diseases which can be effectively immunized by vaccines, malaria vaccines are unable to induce a sterile immunity and a

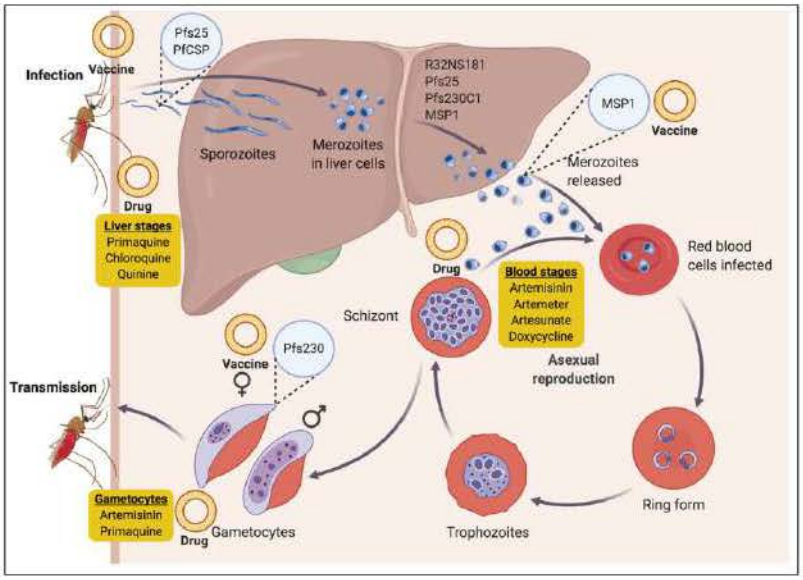

Fig. 3. Summary of drugs and vaccines incorporated in liposomes in the treatment of malaria. Liposomes encapsulate antimalarial drugs and target conserved proteins for vaccines such as PfCSP, Pfs25, Pfs230, and MSP1, inhibiting different stages of malaria infection; liver, blood, and sexual stages (gametocytes).

higher chance of reinfection might occur upon the initial recovery. To date, none of the current vaccines are potent in treating malaria. However, some proteins fractions potential as vaccine candidates.

The sporozoite stage of *Plasmodium falciparum* is layered with circumsporozoite protein (CSP), a vital secreted protein, which interacts with sporozoites adhesion to target cells [171]. A formulated recombinant CSP-based vaccine, known as RTS,S/AS01 vaccine, had manifested potent inhibition of parasites before liver cell replication [172]. Nevertheless, the adverse effect of the RTS,S vaccine is not good in malaria-endemic areas, which, in turn, appeals for liposomal encapsulation [172,173]. In an earlier study, R32NS181 synthetic liposomal encapsulated CSP, had demonstrated a high level of R32-specific serum IgG antibody with minimum systemic toxicity [174]. Pfs25 and Pfs230 antigen fragments (*i.e.*, mostly expressed by the gametocytes sexual stage of human red blood cells) suppressed the transmission cycle and parasite growth in *Anopheles* mosquito [175,176]. These findings highlight the potential of liposomes as a thriving adjuvant strategy in malaria. A summary of drugs and vaccines as candidates to incorporate into liposomes is represented in Fig. 3.

6. Liposomes and HIV

6.1. Liposomes for HIV treatment

Human immunodeficiency virus (HIV) is a blood-borne virus that targets and alters the immune cells. Three stages of HIV infection may include; i) acute HIV infection, ii) chronic HIV infection, and iii) acquired immunodeficiency syndrome (AIDS) [177]. Infection of HIV continues to be a major burden to society worldwide as it claims millions of lives every year and exhausts billions of dollars of resources to support people with HIV [178]. Infection of HIV destroys the host CD4 T cells thus debilitating the immune system and eventually causing AIDS. The structure of HIV is generally complex, encompassing a 9-kilobase HIV RNA which encodes nine genes, yielding 15 distinct proteins. The virus is persistent, leading to chronic infection, and continues to evolve in the infected host, evading the antibody- and cytotoxic lymphocyte- (CTL) responses induced [179].

Currently, there is no therapy that eradicates HIV infection. However, antiretroviral therapy (ART), the gold standard treatment of HIV infection, greatly reduces the viral replication to extremely low levels and allows restoration of the host immune system [180]. ART must be taken correctly in order to suppress the viral infection and several approaches to greatly improve current ART is widely investigated. Liposomes, with their advantageous properties, can be exploited as carriers of therapeutic compounds, as an immune modulator, or even as an adjuvant in vaccine candidates, for HIV-infection prevention and management (Fig. 4).

6.2. Liposomal-based conventional therapy products for HIV

There are several classes of HIV drugs based on their mechanism, including reverse transcriptase inhibitor (both nucleoside and non-nucleoside), protease inhibitor, fusion, and attachment inhibitor. Usually these drugs are taken in combination. A combination of ART drugs, nevirapine, and saquinavir, which is conjugated with liposomes is shown to significantly inhibit viral proliferation compared to free drugs alone [181]. The liposomes conjugated with anti-CD4 would selectively deliver ART drugs to the HIV-infected cells through the CD4 receptor, thus efficiently blocking the viral proliferation with a lower concentration of drugs, possibly reducing drug toxicity and resistance issues. Another antiviral drug, SPC3, which inhibits syncytium formation, is found to be 10 times more potent when encapsulated with liposomes [182]. SPC3 is a synthetic polymeric peptide and contains motifs that correspond to the V3 loop of HIV surface protein. Thus, targeting this

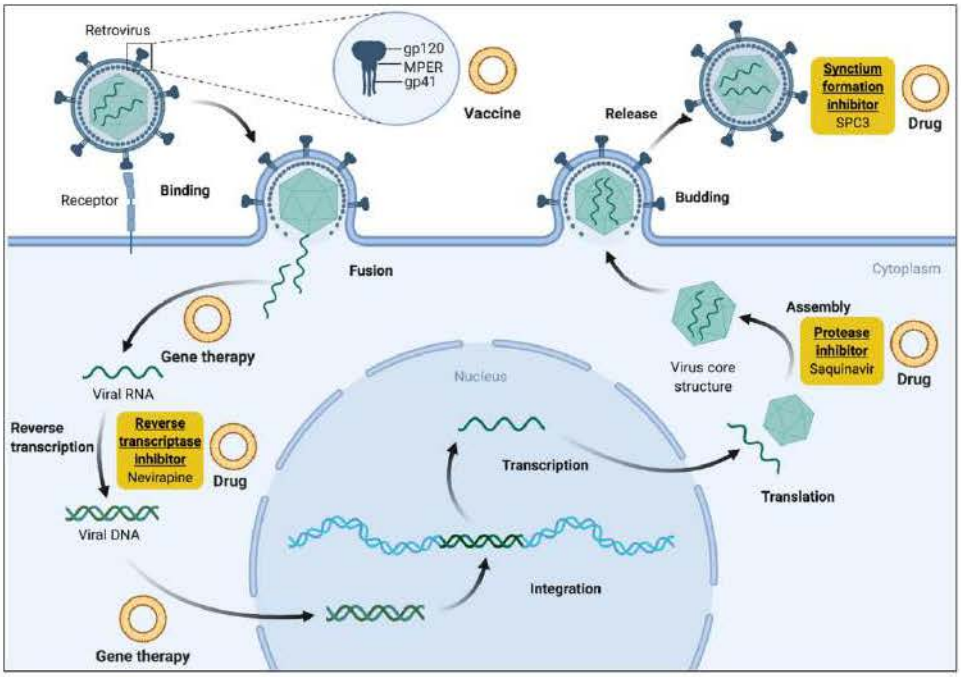

Fig. 4. Liposomes in HIV infection. Liposome-encapsulated ART drugs that inhibit different stages of retroviral replication. In vaccine development, liposomes can function as carriers to vaccine candidates such as gp120, gp41, and MPER and as adjuvants that enhance the vaccines.

domain blocks the lymphocyte and macrophage infection with various HIV strains. However, clinical trials for this antiviral drug only showed moderate effects on viral load. Hence the liposomes are added to improve the effect of SPC3.

Liposome-incorporated HIV drugs delivered as vaginal microbicide products showed a greater efficacy against HSV, HIV, and *Neisseria gonorrhoeae*, compared to the conventional formulation [85]. In addition, liposomes are used for gene therapy approaches to HIV infection. Gene therapy aims to block infection in target cells by allowing viral clearance from carriers or by preventing infection in newly exposed individuals, thus eliminating the latent viral reservoirs in HIV-infected persons and the need for lifelong ART drugs [183]. Gene therapy which includes intrabodies, antisense, and inhibitory RNA therapy, is a promising concept in the prevention of HIV infection as well as in the generation of life-long immunity in infected people. Effective delivery can be achieved by liposomes that specifically bind to HIV-1 and alter the infectivity of this virus to its target cells. Liposomes also are used to deliver anti-HIV drugs to infected cells [184,185]. Transfection of both HIV and diphtheria toxin A fragment gene *in vitro* by cationic liposomes effectively inhibited virus production, enabling the potential of liposomes in HIV gene therapy [186]. In another study, liposome-encapsulated viral envelope region antisense RNA displayed an anti-HIV effect *in vitro*, showing cell-binding of up to 4000–7000 RNA molecules in targeted liposomes and also provides enhanced intracellular half-life [187]. The same observations are supported by several other studies that show encapsulated antisense oligonucleotides in pH-sensitive liposomal formulations displaying an additional enhanced anti-viral effect [188,189].

6.3. Liposomal vaccines for HIV

While ART is the standard treatment against HIV infection, only a portion of eligible people received the therapy. Suboptimal adherence, toxicities, resistance patterns, and drug interaction pose additional obstacles. Hence, vaccines are identified as the most promising approach to controlling HIV infection. In vaccine development for HIV infection, liposomes acts as adjuvants (CAF01, ALF) [190,191]. The liposome is shown to be a more potent adjuvant in HIV vaccine development compared to the conventional aluminium. Using liposomes, HIV-specific cellular responses are induced (both Th1 and Th2-type immunity) and high titers of IgG produced are maintained for a longer period [192–194]. Furthermore, liposomes have the potential to carry antigenic peptides of HIV to elicit potent CTL responses. Previously, lipofectin, a cationic liposome, is demonstrated to effectively deliver HIV-1 Gag, Pol, and Env proteins to dendritic cells and stimulated greater anti-HIV-1 memory CTL responses *in vitro* [195].

The membrane-proximal external region (MPER) from gp41, a subunit on the viral envelope, is identified as the most promising antigen to be utilized in a HIV vaccine. This peptide is the only linear B-epitope that contains an HIV vulnerability site, but this peptide is poorly immunogenic and requires assistance for their display on membrane to achieve effective conformation of the native virus [196]. Liposomes have been used to protect and specifically display this MPER peptide in HIV vaccine candidates [197,198]. This MPER peptide encapsulated with

Table 4
Application of liposomes as DDS and current vaccine development in TB.

Liposomes as drug delivery system (DDS)					
Liposomal encapsulated drug	Type of liposomes	Platform	Observed effects/advantages	Limitations	References
Liposomes encapsulating RIF, INH, and pyrazinamide drugs	Neutral liposomes	*In vivo*	• Prolonged drug accumulation in lungs • Good entrapment efficiency	Lack of observation on macrophage uptake profile of formulations and the targeting activity of liposomal formulations on infected animal models	[124]
		In vitro	• Prolonged drug released • Prolonged sustained drug release		
Dry powder/freeze-dried liposomes encapsulating RIF	Freeze-dried liposomes	*In vivo*	• Prolonged retention time in both oral and pulmonary routes • Enhanced drug internalization in alveolar epithelium	–	[109]
		In vitro	• Good entrapment efficiency • Slow and maximum drug release • Enhanced anti-tubercular activity		
Liposomes containing oxadiazole derivatives from isoniazid (INH)	Natural phospholipid liposomes	*In vivo*	• Decreased pulmonary bacterial load • High drug-release profile • Less tissue damage in lungs	Oxadiazole derivative is not a prodrug because INH derivatives cannot be expected to overcome INH-resistance, due to that the molecular action mechanism is identical	[125]
N' Dodecanoullisonicotinohydrazide (DINH) loaded liposome-in-hydrogel	Natural phospholipid liposomes	*In vivo*	• Stable drug concentration and duration • No obvious cytotoxicity • Longer localized sustained drug release	–	[126]
		In vitro	• Slower initial burst release for effective anti-tubercular activity • Prolonged sustained drug release		
RIF-loaded liposome embedment in a polymer matrix	Natural phospholipid liposomes	*In vitro*	• Prolonged drug release	Lack of observation on simultaneous release of two or more drugs with different biomedical properties in the same system.	[127]
Co-encapsulation of RIF and INH into crude soybean CL liposomes	Crude soybean lecithin liposomes	*In vitro*	• Efficient encapsulation • Prolonged drug release	Lack of *in vivo* testing	[128]
Liposome encapsulating phospholipid cardiolipin and fluoroquinolone LF	Natural phospholipid liposomes	*In vitro*	• Increased anti-tubercular activity of cardiolipin	–	[130]

Liposomes in vaccines development					
Targeted protein incorporated into liposomes for vaccines	Type of liposomes	Platform	Observed effects/advantages	Limitations	References
Liposomes with DDA/TDB	Natural phospholipid liposomes	*In vivo*	• Effective induction of Th1 responses • Increased IL-17 • High IgG2/IgG1 ratio	Lack of observation on *M. tuberculosis*-infected mouse challenge model	[131]
Formulating *M. tuberculosis* BCG in DDA/TDB	Natural phospholipid liposomes	*In vivo*	• Similar BCG Pasteur profile with conventional BCG immunization • Elevated BCG bacilli in lymph nodes • High T cells responses	Lack of observation on the relationship between post-BCG vaccination lymph node involvement and the generation of an effective anti-tuberculosis protective response	[132]
Mycobacterium bovis BCG formulated in dimethyl dioctadecyl ammonium bromide (DDA) – D (+) trehalose 6,6 dibenenate (TDB) adjuvant	Cationic liposomes	*In vivo*	• Reduced bacterial load in lungs and spleen • reduction in the number of lesions and severity of pathology	Lack of observation on the nature of the cellular immune response of the guinea pigs to glycolipids to confirm specific CD1-restricted response against the microbial antigenic lipid compartments	[134]
Phosphatidylserine liposomes encapsulated Ag85B and ESAT-6 TB antigens	Natural phospholipid liposomes	*In vitro*	• Mixed Th1/Th17-Th2 immune response for Ag85b, weak response to ESAT-6 • Reduced bacterial load in lungs and spleen • Accumulation of memory T cells in lungs	–	[135]
dLOS/DDA liposomal adjuvant	Cationic liposomes	*In vivo*		–	[136]

(continued on next page)

Table 4 (*continued*)

Liposomes in vaccines development					
Targeted protein incorporated into liposomes for vaccines	Type of liposomes	Platform	Observed effects/advantages	Limitations	References
			• Long-term storage of subunit TB vaccine at the injection site		
		In vitro	• Enhanced interaction with APCs • Induced both humoral and cellular immune responses • Induced strong memory response		

Table 5
Application of liposomes as DDS and current vaccine development in malaria.

Liposomes as drug delivery system (DDS)					
Liposomal encapsulated drug	Type of liposomes	Platform	Observed effects/advantages	Limitations	References
Chloroquine encapsulated PEGylated liposomes	Natural phospholipid liposomes	*In vivo*	• Enhanced the clearance of blood parasites • Significant delay in death of mice • Increased its maximum tolerable doses	–	[146]
Platinum-CQ diphosphate dichloride-PEGylated liposomes	Natural phospholipid liposomes	–	• Higher entrapment efficiency • Minimum leakage within 2 months of storage • Delayed drug release	Lack of *in vivo* and *in vitro* testing	[147]
Primaquine-diphosphate (egg yolk phosphatidylcholine, bovine phosphatidylserine, and cholesterol) liposomes	Natural phospholipid liposomes	*In vivo*	• Low toxicity levels • Slower elimination of drug	–	[150]
Primaquine-diphosphate (phosphatidylcholine phosphatidylserine, and cholesterol) liposomes	Natural phospholipid liposomes	*In vivo*	• High bioavailability of drug at the targeted organ	–	[156]
Primaquine + chloroquine (PQ-CQ) (HSPC, cholesterol and DSPE-mPEG) liposomes	Natural phospholipid liposomes	*In vitro*	• Enhanced efficacy and reduced toxicity • Delayed drug release • Lower drug encapsulation	Further details on pharmacokinetics profile were not fully discussed	[157]
Artemisinin-PEGylated liposomes	Natural phospholipid liposomes	*In vivo*	• Efficient encapsulation (>70 %) • Prolonged half-life time	A slower rate of clearance from peritoneum area	[173]
Artemisinin-PEGylated liposomes	Natural phospholipid liposomes	*In vivo*	• Extend the half-life of artemisinin • Higher encapsulation efficiency	Long-term administration was not discussed	[167]
Artemisinin-conventional and PEGylated liposomes	Natural phospholipid liposomes	*In vivo*	• Prolonged circulation • Immediate antimalarial effect	–	[209]

Liposomes in vaccines development					
Targeted protein incorporated into liposomes for vaccines	Type of liposomes	Platform	Observed effects/advantages	Limitations	References
R32NS181 encapsulation into monophosphoryl lipid A liposomes	Synthetic liposomes	*In vivo*	• High level of R32-specific serum IgG antibody • Minimum systemic toxicity	–	[204]
Pfs25 incubated with cobalt-porphyrin-phospholipid (CoPoP)/ PHAD liposomes	Synthetic liposomes	*In vivo*	• Enhanced strong transmission-reducing activity	–	[175]
Pfs230 incubated with cobalt-porphyrin-phospholipid (CoPoP) liposomes	Synthetic liposomes	*In vivo*	• Elicitation of IgG antibodies	–	[176]

liposome not only showed high production of MPER-specific antibodies but also exhibited a durable humoral response *in vivo* [199].

Another antigen identified, gp120, can be inhibited by several molecules (macrocyclic peptide, N-butyldeoxynojirimycin), shown inhibit viral cell interaction with nanomolar potencies [200]. As a candidate in vaccine development for HIV, these peptides are encapsulated with liposomes to slowly release the inhibitor for long-term protection against HIV infection. They demonstrate additional antiviral effects (reduce viral secretion, neutralize free viral particles) [201,202]. Extensive investigations and promising observations of liposomes use in the management and treatment all these major infectious diseases such as DDS and vaccine development (Table 4, Table 5 and Table 6) indicate the true potential of this nanomaterial to be further developed into marketable therapies.

7. Conclusion

Recent advances in nanotechnology have opened up the potential of

Table 6
Application of liposomes as DDS and current vaccine development in HIV.

Liposomes as drug delivery system (DDS)					
Liposomal encapsulated drug	Type of liposomes	Platform	Observed effects/advantages	Limitations	References
iLPs (egg PC: cholesterol:DSPE-PEG) conjugated with nevirapine and saquinavir	Natural phospholipid liposomes	*In vitro*	• Inhibit viral proliferation with a lower dosage	• Dose-dependent cytotoxicity	[181]
Liposomes-associated SPC3	Natural phospholipid liposomes	*In vitro*	• 10-fold inhibition of HIV-induced fusion • Improved anti-viral efficacy	• Low stability	[182]
Liposomes in vaginal microbicide	Natural phospholipid liposomes	*In vivo*	• Improved dose level • Absence of toxicity	• Excipients in formulation reduced activity against HSV and HIV	[183]
		In vitro	• Minimum toxicity of lactobacilli		
HIV-DT-A- cationic liposome	Natural phospholipid liposomes	*In vitro*	• Co-transfection of DT-A effectively inhibits virus production	• Low efficiency of transfection • High toxicity towards HeLa cells	[196]
Liposome encapsulated antisense RNA	Natural phospholipid liposomes	*In vitro*	• Suppressed tat gene expression (90 %) and gp160 production (100 %)	–	[197]
pH-sensitive liposomes carried antisense HIV	Natural phospholipid liposomes	*In vitro*	• Effectively delivered antisense oligonucleotides into HIV-infected macrophages	• Susceptible toxicity towards HIV-infected macrophages	[198]
Cationic liposome-encapsulated antisense HIV-1 gag	Cationic liposomes	*In vitro*	• Effectively inhibited p24 antigen production	–	[199]
		In vivo	• Enhanced cellular uptake for anti-HIV activity		

Liposomes in vaccines development					
Targeted protein incorporated into liposomes for vaccines	Type of liposomes	Platform	Observed effect/advantages	Limitations	References
Lipofectin	Cationic lipid	*In vivo*	• Enhanced specific memory CTL response	• Instability of liposomes	[195]
Trirepeat of ELDKWA sequence of gp41 antigen with defensins in liposomes	Natural phospholipid liposomes	*In vitro*	• Higher IgG/IgA titer in serum and mucosal washes	–	[199]
Macrocyclic peptide triazoles (cPTs) AAR029b encapsulated in PEGylated liposomes	Natural phospholipid liposomes	*In vivo*	• Substantial half-life time • Reduced clearance rate	–	[200]
Liposomes-based adjuvant CAF01	Synthetic liposomes	*In vitro*	• Similar induction of CTL responses as alum	• Self-aggregation of individual lipid constituents	[190,191]

liposomes to address several limitations in existing therapeutic and prophylactic approaches to infectious diseases. In fact, among the many types of nanomaterials, liposomes have potentially broad applicability due to its advantageous properties; they are biodegradable, biocompatible, and versatile. This is evidenced through the numerous liposomal formulations that were approved for indications in several diseases. Other liposome-based formulations are undergoing clinical trials including the infamous CAF01, a liposomal adjuvant candidate for vaccines. In infectious diseases, liposomes are employed as tools in drug delivery systems and vaccine development. Although currently there is no liposomal system that have reached approved status for indications in infectious diseases, there is great potential for the success of liposome-based therapies in this field as long as its several challenges that limit its translation into clinical practise are strategically addressed. This includes enhanced permeability retention effect, liposomes design and its payload release, the overcoming of immune system barriers and solving the inconsistencies between clinical results and experimental models. The emergence of new studies will present many new opportunities for liposomes to further increase their therapeutic efficacy as part of a future paradigm in infectious diseases treatment.

Funding

This work was supported by grants comprising the Fundamental Research Grant Scheme (FRGS) (FRGS/1/2018/SKK08/USM/03/1), Ministry of Higher Education (Malaysia) and the Long-Term Research Grant Scheme (LRGS) (LRGS/1/2015/USM/01/1/1) by the Ministry of Health Education (Malaysia).

CRediT authorship contribution statement

ASNA, SA, and SMNH conceived and designed the manuscript, performed a literature search, and wrote and revised the manuscript. NAAS, SA, and SMNH prepared the figures and table. MES, AA, RM, JI, and RK conceived and revised the manuscript. RK acquired the funding for this work. All authors contributed to the paper and approved the final draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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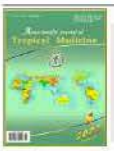

Review Article

Asian Pacific Journal of Tropical Medicine

apjtm.org

doi: 10.4103/1995-7645.332806 5-Years Impact Factor: 2.285

Liposomes as immunological adjuvants and delivery systems in the development of tuberculosis vaccine: A review

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ABSTRACT

Liposomes are phospholipid bilayer vesicles, which are biocompatible, biodegradable and nontoxic vehicles suitable for numerous drug and gene delivery applications. In this review, we discuss the prospect of using liposome technology in the development of a vaccine for tuberculosis. Tuberculosis remains an important health problem that requires the development of an effective vaccine, especially since the only approved vaccine for it continues to be the Bacille Calmette-Geurin (BCG) one developed 100 years ago. This review focuses on the different applications of liposomes toward achieving this goal. Numerous liposomal formulations showing prospect in the research stage and in clinical trials are discussed.

KEYWORDS: Liposomes; Tuberculosis vaccine; Adjuvant; Delivery system; Tuberculosis

1. Introduction

According to the World Health Organization (WHO), tuberculosis (TB) together with human immunodeficiency virus (HIV) is one of the most critical diseases and causes of mortalities in adults worldwide. TB imposes a significant economic burden on most countries around the world[1]. Although there has been a decrease in the global annual death rate due to TB during the last 15 years, this bacterium still produces an alarming number of 1.5 million deaths per year[2]. Another important aspect of the TB pandemic is the growing appearance of strains with antibiotic resistance[3]. Multidrug-resistant TB (MDR-TB) is defined as TB resistant to common drugs, such as isoniazid or rifampin. MDR-TB is estimated to currently affect 480 000 people annually, with only half of these patients receiving appropriate treatments[4]. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, may be undetectable in the lung and survive in it for prolonged periods of time in a dormant state, which makes the process of diagnosis and timely treatment difficult. Latent TB infection and the reactivation of infection can happen at any time, particularly following the immune compromise[4,5].

Vaccination is the most desirable means of preventing TB. French scientists Albert Calmette and Camille Guérin developed the Bacille Calmette-Geurin (BCG) vaccine in 1921. Exactly 100 years later, this remains the only licensed human vaccine against TB and has been used all across the world for more than 80 years[6]. BCG is a live attenuated strain of *Mycobacterium bovis* and is used in 80% of the TB endemic areas[7]. Although BCG can prevent dissemination of TB in children, its protective effect is variable and questionable in adults[8].

The advent of new technologies in vaccine development, including new adjuvant formulations, the whole-genome sequencing of Mtb, and other mycobacteria, has been used to define new TB vaccine candidates[9]. Due to the evasive nature of Mtb during the infection process, the use of the bacterial components, such as lipids, has been

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How to cite this article: Luwi NEM, Ahmad S, Azlyna ASN, Nordin A, Sarmiento ME, Acosta A, et al. Liposomes as immunological adjuvants and delivery systems in the development of tuberculosis vaccine: A review. Asian Pac J Trop Med 2021; 15(1): 7-16.

Article history: Received 2 May 2021 Revision 26 May 2021
Accepted 6 December 2021 Available online 20 January 2022

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studied intensely by the researchers as one of the components for TB vaccine development[10]. Here, the use of liposomes derived from the lipid components of the bacterial cell has been considered as a special prospective vaccine candidate against the TB infection due to their capacity to induce strong humoral and immune responses[11]. In what follows, this approach to TB vaccine development will be reviewed and discussed.

2. Mechanisms of infection

TB is a major lung disease and the Mtb bacterium is the etiological agent[12]. The main port of entry for the bacterium is the respiratory tract. Upon reaching the lung, Mtb becomes engulfed by phagocytic cells such as macrophages and dendritic cells (DCs)[13]. The first-line defence against Mtb infection is the innate immune response, which is represented by macrophages, DCs, and natural killer (NK) cells among others. These cells are activated by interaction between the pathogen associated molecular patterns (PAMPs), such as glycolipids, lipoproteins, and carbohydrates on Mtb and the pattern recognition receptors (PRRs) on the host cells, which include Toll-like receptors, NOD-like receptors, and C-type lectin receptors[13,14]. This presence on the infected cells allows for the recognition of the pathogens, the reactivation of the effector cells, and the uptake of Mtb by phagocytic cells[13].

After the activation of the innate immune response, a specific immune response is produced against Mtb. This response is represented by T helper (Th) $CD4^+$ and T cytotoxic $CD8^+$ cells and accompanied by the production of specific antibodies[15]. Mtb, however, employs an effective strategy to evade both the innate and the adaptive immune responses[16,17]. With respect to the innate immunity, it inhibits apoptosis and triggers necrosis of host macrophages, which delays the initiation of adaptive immunity[18]. The manipulation of macrophage death pathways is one of the mechanisms used by Mtb to evade host defences[18]. In addition, Mtb uses various mechanisms to inhibit pathways for antigen presentation to T cells[13]. The evasion mechanisms allow Mtb to establish a persistent or latent infection in macrophages, which results in inhibition of major histocompatibility complex class II (MHC-II) molecule expression and antigen presentation[19]. This ability of Mtb to inhibit MHC-II antigen presentation leads to inhibition of recognition of $CD4^+$ T cells. It is important to note that immunity to TB depends on $CD4^+$ T cells for the control of primary infection and it is essential for ongoing immune surveillance to control the infection that forms the reservoirs for reactivation of TB[19,20].

3. Liposomes

Liposomes are relatively small spherical vesicles whose membranes consist of one or more phospholipid bilayers (Figure 1). Liposomes were first reported by Bangham *et al*. in 1965 and their use has been established in several medical areas of interest, including the oral delivery of vaccines, insulin administration and cancer chemotherapy[21–23]. Liposomes have important biological and technological advantages over many other types of medication carriers and have been used with success as delivery systems for biological substances both *in vitro* and *in vivo*[24]. The efficacy of liposomes as carriers of drugs is partly due to their capacity to release the medication cargo in target cells[25]. The interest in the use of liposomes has also been tied to specificities of their composition, which is biodegradable and biocompatible[26]. They can be produced using natural or synthetic lipids and take the form of concentric bilayered vesicles in which an aqueous volume is entrapped[27]. The range of sizes of liposomes enables them to reach the targeted cells, including antigen-presenting cells such as DCs[28]. Several studies have shown that the size of liposomes depends on the preparation method[29]. The smaller sized, 50-250 nm unilamellar vesicles (ULVs) are customizable for encapsulation of hydrophilic drugs and have a longer half-life when compared to multilamellar vesicles (MLVs) with size ranging from 500-5000 nm[27]. Another important property is their capacity to protect entrapped bioactive materials (*e.g.*, hydrophilic and hydrophobic drugs) from immediate degradation, thus increasing the efficacy of drugs and decreasing their toxicity[29,30]. Liposomes have been used to facilitate the cellular uptake of the drugs directly by the targeted cells such as macrophage and DCs[31].

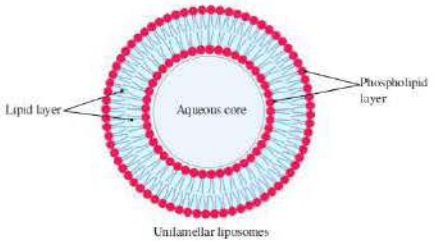

Figure 1. Basic structure of liposomes.

3.1. Synthesis of liposomes

The earliest liposome was discovered and synthesized by Bangham and colleagues using thin layer hydration (TLH) techniques, also known as Bangham techniques[22]. Due to promising advantages of liposomes in biomedical and biotechnology areas, many researchers started to produce liposome using conventional or supercritical-assisted techniques. The conventional techniques of liposome production has occurred earlier than supercritical-assisted techniques, which can performed at room temperature and high pressure system. The size of liposomes produced ranged from 1 μm up to 300 nm with poor stability, whereas the encapsulation efficiency ranged between

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20% to 90%[32,33]. Supercritical-assisted techniques is a rapidly evolving modern and green technology for expanding the production of vesicles on a large scale. The size of liposomes ranged between 20 nm and 300 nm with better dimensional control as deduced from the liposomes heterogeneity measure known as polydispersity index (PDI), and the higher encapsulation efficiency of up to 90%[32–35]. Liposomes can be tailored to possess different sizes, ranging from very small (0.025 μm) to large (2.5 μm), and can be produced using different methods[29]. Different methods of synthesis of liposomes can lead to a variety of sizes and numbers of liposome bilayers[36]. Accordingly, liposomes can be classified according to their size and phospholipid bilayer number (Figure 2).

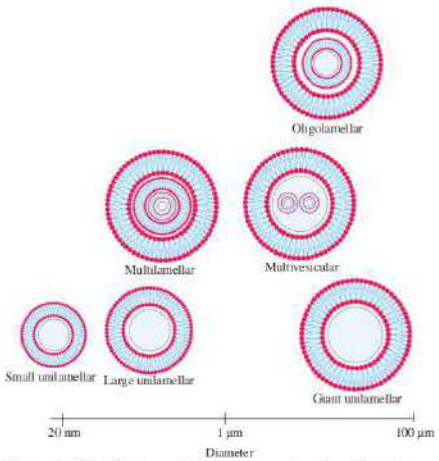

Figure 2. Classification of the liposomes based on their size and phospholipid bilayers.

3.2. Physiochemical properties of liposomes

Liposomes have been used in analytical, diagnostic and therapeutic applications owing to their unique physiochemical properties[37]. One of the important elements reported with respect to the physiochemical properties of liposomes is the phospholipid bilayer. The bilayer composition of liposomes allows the interaction with the biomolecules, such Doxil® and Depocyt® and both of these biomolecules have been used clinically so far in cancer therapy[38,39]. Different ligands such as peptides, proteins, monoclonal antibodies, and carbohydrates can be coupled to the surface of liposomes, leading to the interaction with the specific target cells, which promotes an increase in the therapeutic efficacy[38]. The addition of cholesterol to the lipid bilayer can increase their stability *in vivo* and *in vitro*[24]. Apart from that, the aqueous interiors of liposomes can be incorporated with hydrophilic and/or amphiphilic drugs, which have been used with success in targeted cancer therapy[40].

The vesicle size and the bilayer structure are the two most important factors determining the physiochemical properties of liposomes and they greatly influence the liposomal vaccine design[41]. If these two properties are appropriately tuned, liposomes can pass through the tumor vessels and concentrate in the target site. Previous studies have found that liposomes with a size smaller than 100 nm in diameter can circumvent the capture by the reticuloendothelial system (RES), have a longer half-life in blood and accumulate in the tumoral site[42]. In contrast, liposomes of larger sizes did not escape the RES uptake and got eliminated rapidly from the blood circulation[42]. Vesicle lamellarity also influences the immune response against liposome-associated antigens[41]. A previous study investigated the small unilamellar vesicles (SUVs) with no TLR agonist and showed a higher capacity to induce the spleen IFN-γ response against Ovalbumin (OVA) compared to multilamellar vesicles (MUVs)[43]. The ability of SUVs to induce a potent CD8 T cells response shows that SUV is the preferred state to potentiate innate and adaptive immune responses for an improved vaccine efficacy[43,44].

3.3. Advantages of liposomes

Liposomes have been currently approved as adjuvant/antigen delivery agents and exploited to deliver therapeutic compounds and immunomodulators for a broad range of diseases[45]. It has been reported that liposomes can incorporate antigens and targeting molecules to serve as potent vaccines[46]. In contrast to liposomes for the delivery of cancer drugs, liposomes that get rapidly cleared from the blood through elimination by phagocytic cells, hepatic Kupffer cells, and macrophages, alongside being uptaken by the target cells, may be particularly suitable for the application in vaccines[42]. One such prompt presentation to the cells of the immune system will increase the delivery efficiency and invoke less of the systemic toxicity in the organism[47]. Here it should be noted that liposomes produce less toxicological effects when they are injected in low doses[47]. Moreover, liposomes also do not cause an antigenic reaction by themselves[35], which makes them suitable for the role of carriers of antigenic loads.

There are many benefits of liposomes that stem from their structure and composition. Liposomes have the ability to facilitate the binding of the target to the liposomal surface, which make them a suitable candidate for drug delivery in general, but they are also capable to increase the therapeutic efficacy of the drug[29]. Encapsulation of drugs in liposomes improves the pharmacokinetics and also protects the drug against deactivation, which has been exploited in the delivery of unstable antibiotics[48]. For this reason, encapsulation of ciprofloxacin in SUVs significantly improved the antibacterial activity against Francisella tularensis infections[49]. With this many advantages, further studies into liposomes and their application in various fields of medicine must be delved into.

3.4. Immunostimulatory properties of liposomes

Because of their unique properties and advancements in their

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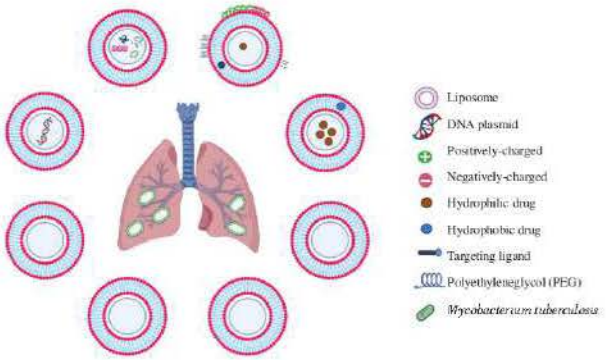

Figure 3. The use of liposomes in development of *Mycobacterium tuberculosis* vaccine.

synthesis, liposomes have been widely used to stimulate the immune response. The enhancement of the immune response is dependent on the cellular uptake of liposomes by the target cells. However, the cellular uptake of liposomes is affected by their surface charge. A previous study found that the surface charge of liposomes influences the cellular uptake through the endocytotic pathway in glioblastoma cells[50]. Meanwhile, the pharmacokinetic properties of liposomes can have a significant effect on the efficiency of the presentation of liposomes to target cells such as DCs, macrophages, and other cells[24,31]. DCs are professional antigen-presenting cells that can express cytokines, co-stimulatory molecules that regulate the primary immune response. In addition, DCs are considered phagocytic cells, too, since they have the ability to take up substances in peripheral tissues, undergo maturation and promote the T cell response[31]. DCs will process the antigens and present them on the cell surface to T lymphocytes[51,52]. Once liposomes are stably surface-modified and functionalized, they can be conjugated with several molecules such as polyethyleneglycol (PEG) and antigens to specifically target DC receptors to enhance the uptake and initiate the selective adaptive immune response[28].

Apart from DCs, liposomes also can be associated and uptaken by other target cells such as macrophages[31]. As a particulate drug carrier, liposomes will naturally target the mononuclear phagocytic system. Mononuclear phagocytic cells express a range of receptors such as scavenger receptors, mannose receptors, and integrins that can be targeted by ligands of liposomes, thus increasing the target specificity toward the macrophages. This advantage for targeting macrophages using liposomes can lead to cell activation for the treatment of chronic inflammation and cancer[31]. Besides, the addition of antigens in formulated liposomes could enhance the activation and uptake capacity of DCs, which increases the ability of DCs to induce T cell proliferation[53]. A previous study found that mannosylated liposomes cause a high expression of surface markers and stimulation of T cells[54], which suggests that they can represent a versatile delivery vehicle to enhance the immune response.

The lipid surface of liposomes can be chemically modified to increase the circulation time, accumulation time at the target site, and cellular internalization[55]. In a previous study, liposomes were modified and made multifunctional by adding functional groups to their surface. The addition of these functional groups increased the longevity of liposomes in blood, favouring the specific targeting in response to the local stimuli at the target site. Active targeting of liposomes has also been achieved by conjugation with peptides or ligands to reduce the interaction with off-target cells[55,56]. Liposomes with specific surface ligands actively targeted and interacted with cancer cell surface receptors in the tumor environment, which led to enhanced uptake and therapeutic effect at the tumor site[56]. Other studies have found that liposomes are able to target the endoplasmic reticulum (ER) and associated membranes specifically[57]. Once liposomes are inside the ER, the targeting lipid intercalates with the ER membrane and incorporates itself into ER-assembling entities such as lipid droplets and secreted proteins[57]. These ER-targeting liposomes were found to be effective for prolonged delivery of lipids and lipophilic drugs into human cells[55,57,58].

4. Liposomes in TB vaccine development

Liposomes are versatile and widely used as an efficient adjuvant and delivery system (Table 1). Although the majority of their applications as agents for the delivery of immunostimulatory molecules have been in the domain of cancer immunotherapy, they can be considered as an ideal vaccine carrier candidate, especially for TB (Figure 3). This application will be reviewed in the following section in detail.

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Table 1. Summary of liposomes in tuberculosis vaccine development.

Adjuvant	References	Delivery strategy	References
CAF01	[67-76]	Passive targeting	[88-91]
AS01	[2,78,79,106-109]	Active targeting	[37,44,91,92]
DOTAP	[80-82]	Nucleic acid- Ag85A	[37,93-95]
AdHu5Ag85A	[83-86]	Live attenuated and killed whole cell vaccines-RUTI	[98-103]

4.1. Liposomes as adjuvants

The strategy to develop TB vaccine has resulted in several different liposome-based adjuvant candidates. The word 'adjuvant' comes from the Latin word 'adjuvare', meaning 'to help' or 'to enhance'[59]. The development of liposome-based adjuvants in TB vaccine formulations is indeed intended to enhance the immune responses against Mtb antigens[60]. In recent years, it has been shown that cationic liposomes in combination with other immunostimulatory factors such as TDB, MPL, and Poly I : C can induce a solid immune response against Mtb antigens[11]. Cationic liposomes provide long-term storage for subunit TB vaccines at the injection site and have been shown to provide a potent surface charge when interacting with APCs to promote both humoral and cellular immune responses[11,44]. Cationic liposomes have been combined with dimethyldioctadecylammonium (DDA) stabilized with glycolipid immunomodulator Trehalose 6,6-dibehenate (TDB), which is a synthetic variant of the cord factor located in the mycobacterial cell wall[61]. The combination of these DDA-TDB liposomes with the mycobacterial fusion protein Ag85B-ESAT-6 is a novel TB vaccine candidate for CAF01. The stabilizing properties of CAF01 make it suitable for use in vaccine formulations and their safety was demonstrated in a Phase I trial. Vaccination with CAF01 resulted in highly complex immune responses with strong T cell immunity, which indicated that CAF01 might be a good candidate for future TB vaccine development[62]. Knudsen and colleagues performed a detailed comparison of five different clinical adjuvants, including CAF01, and showed a mixed Th1/Th17 profile in mice[63]. In TB, Th1 immune response is required against Mtb infection, while Th17 immunity was rapidly induced upon Mtb infection, conferring protection similar to vaccination[63,64]. The mycobacterial phospholipid from *Mycobacterium bovis* BCG lipid extract was shown to induce a potent Th1 immune response, characterized by an increase in the expression of IgG2a and IFN-α[65]. Further investigations showed that the formulation with a liposomal adjuvant is a promising system that successfully induced a prolonged uptake and activation of DCs to elicit Th1 and Th17 cells in both neonates and adults[66,67]. This adjuvanted vaccine candidate with CAF01 manages to induce a robust multifunctional memory in T cells, which is maintained for over a year post-vaccination[68]. Durable protection via T cells induced by CAF01 formulation preferentially localized to the lung site of infection[69]. Recently, incorporation of additional immunomodulatory adjuvants such as monophosphoryl A and Pam3Cys as recognition agonists into the CAF01 formulation led to new liposomal adjuvants. These new formulations effectively induced a specific immune response against the mycobacterial DNA and antigen, respectively, and they also provided an enhanced and persistent protection against the Mtb infection[2]. Other studies also found that CAF01 in combination with the anti-subunit TB vaccine, H56, results in an increased response towards polyfunctional CD4 T cells that localize to the lung parenchyma. This leads to prolonged and sustained protection to infected antigen presentation cells in mice and to date this study is still in clinical development[69,70]. These findings demonstrated liposomes not only to be a viable vaccine carrier that induces long-term protection against Mtb, but also a safe and tolerable adjuvant producing no adverse or systemic effects observed upon vaccination, notwithstanding that further more in-depth safety profiles need to be established[71].

Liposomes have been studied in comparison with alum and oil-in-water emulsions as adjuvants for TB vaccines. Preclinical studies have shown that both emulsion-based and liposome-based adjuvants provide protection against mycobacterial challenge[2]. AS01 is a liposome-based adjuvant vaccine containing two immunostimulants: 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) and saponin QS-21[72]. This liposome-based adjuvant such as AS01 caused the rapid effect after being localized to the injected muscle and draining the lymph node. Besides, AS01 also triggered a higher $CD4^+$ T cell response, as indicated by a stronger IFN-γ response, confirming that it is a suitable candidate for an adjuvant in a TB vaccine[2,72,73]. As far as the clinical trial was considered, this adjuvant system was used in the form of M72 antigen. M72-AS01E (E referring to reduced dose for pediatric use) is currently in Phase 2b clinical trial and is deemed safe in healthy adolescents and adults[74,75]. It induces immunogenicity and protection in adults with active and latent Mtb infection[76,77].

Another lipid tested as a liposomal adjuvant in an animal challenge study is 1, 2-dioleoyl 3-trimethylammonium propane (DOTAP). DOTAP is a cationic liposome-forming compound that has been used as an adjuvant in TB vaccine development. It acts as a booster of the immunogenicity of peptide and protein antigens to produce Th1 immune responses[62,78]. DOTAP in combination with a fusion protein of Mtb HspX, PPE44, and EsxV elicits a strong immune response. Mice that received this combination of DOTAP secreted more IFN-γ and IL-12, indicating a strong Th1 response[78]. Apart from that, a previous study has shown that DOTAP exhibits mucosal adjuvant effects when ovalbumin (OVA) combined with DOTAP is delivered via the intranasal route, as indicated by the strong Th2 immune response[79]. However, further development of DOTAP as a mucosal adjuvant for vaccination against TB is still needed.

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The current liposomal adjuvants, in fact, are most appropriate for injection. Still, the potential for stimulating an immune response combined with the possibility of 'needleless vaccination' has provoked an interest in their use in mucosal vaccination. For example, the protection achieved by the intranasal mucosal immunization with AdHu5Ag85A was associated with the localization of antigen/adjuvant specific T cells to the lung airways[80,81]. A previous study used the rhesus macaque TB model to evaluate the safety effects of AdHu5Ag85A. The results showed that the mucosal boost immunization was safe in BCG rhesus macaque in which an enhanced antigen-specific T cells response was observed[82]. The mucosal route for TB vaccination has already reached Phase 1 clinical trials and the assessment of the levels of protection specific to this adjuvant is underway[83].

4.2. Liposomes as delivery systems

The characteristics of liposomes to entrap substances such as drugs have made liposomes an excellent tool to explore as a new drug delivery system for the development of TB vaccines[25]. Liposomal formulations are able to increase the bioavailability of drugs and reduce the treatment time[84]. Liposomes can also be targeted to specific tissues or organs by active or passive methods. Passive targeting involves the transport of liposomes to the target tissues by tailoring their surface structure to a desired systemic distribution profile. The combination of passive targeting in TB vaccine development with the inhalation route has gained interest of the researchers because liposomes owe their ability to reach alveoli macrophages to an adequate size. Once in the blood circulation, liposomes are easily taken up by phagocytic cells in the mucosal pulmonary systems[84–86]. Apart from that, the development of liposomal formulations for aerosol delivery hints at the potential for their use in intranasal TB vaccines because liposomes can thus reach the lung tissues more effectively[86]. The use of passive targeting has been employed by Gaspar *et al.*, who encapsulated multilamellar vesicles of liposomes with rifabutin and thus achieved a higher concentration of the antibiotic in targeted organs compared to the treatment with free rifabutin[84,87]. Along this line, innovative inhalation therapies with liposomes may contribute to the development of TB vaccines for pulmonary administration.

Meanwhile, for the purposes of active targeting, the phospholipid bilayer of liposomes is coupled to targeting ligands, including peptides, antigens and proteins, so as to make them suitable carriers for the delivery of drugs to specific sites with improved therapeutic outcomes[25,84]. The incorporation of antigens in liposomes influences immunogenicity by inducing T cell response and indirectly increasing the availability for antibody or B-cell recognition[41,60]. Gerald and colleagues found that immunization of liposomal mycobacterial lipid antigens induced protection in guinea pigs challenged with Mtb[88]. The formulation consisting of a liposomal system with mycobacterial lipid antigens reduced the bacterial load in the spleen of inoculated animals as compared to the unvaccinated group of animals. In another study, liposomes based on phosphatidylserine (Lipo-AE) carrying a mycobacterial antigen were shown to induce the accumulation of memory T cells in the lung and reduce the bacterial load in both lung and spleen, thereby boosting BCG immunization[3]. These findings show that liposomes are convement as delivery systems for lipid antigens *in vivo*, in part because their phospholipid bilayers are suitable for incorporation of an amphipathic antigen[88,89].

Nucleic acid vaccines have emerged as alternatives to traditional vaccines in inducing an immune response against TB. Liposomal delivery systems encapsulating DNA plasmids represent the most promising strategy to stimulate the immune responses[60,90]. For example, liposomes encapsulating a *Mycobacterium* DNA and incorporating Ag85A caused a substantial expression of DNA in the mucosal intestinal epithelium as well as in microfold cells, DCs, and Peyer's patches of the small intestine. These cellular compartments play an important role in regulating the immune response[91]. This approach has resulted in oral vaccination with liposomal-DNA Ag85A able to generate antigen-specific mucosal and systemic humoral immunity against TB[90,91]. Furthermore, liposomes incorporating the same DNA showed an enhancement in CD4 and CD8 T cell response and were capable of prolonging survival in mice infected with TB[2,92].

Finally, live attenuated and killed whole-cell vaccines have been studied as candidates for TB vaccines because of their advantages over protein-adjuvant formulations and recombinant viral-vectored ones[93]. Live whole-cell vaccines possess the ability to induce long-lasting memory immune responses by employing a broad antigen composition to stimulate the production of T cells and B cells responses[93]. Nowadays, the live attenuated vaccines have entered the preclinical and clinical developments with the recombinant BCG and attenuated Mtb[94]. One of the liposomal therapeutic vaccine candidates is a vaccine made of fragmented Mtb cells detoxified and liposomed (RUTI), a polyantigenic liposomal vaccine composed of detoxified fragmented Mtb cells. RUTI is developed to prevent active TB in subjects with latent TB infections by boosting the previous immunity through chemotherapy, which has triggered a Th1/Th2 response in infected mice[95,96]. Furthermore, RUTI has been shown to reduce the bacillary load and increase the survival rate of infected animals in short- and long-term vaccination, respectively[97]. This vaccine also facilitated a response of Th cells to a wide range of antigens, along with an increased antibody production, thanks to which it entered Phase 2 clinical trial in 2014[93,98]. The treatment with RUTI appears to be well tolerated and the immunogenicity profile in latent TB infections will be based on a single injection of a highest dose[95]. As preclinical results are similar or even enhanced compared to BCG vaccination, Phase 1 clinical trial of RUTI has indicated that it is a safe treatment option for healthy individuals, as it confidently triggers the specific T cell response against Mtb[99]. This approach of utilizing fragments of Mtb in vaccines has driven

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the field to explore other species of mycobacteria. One of them is *Mycobacterium smegmatis*, a non-pathogenic *Mycobacterium* that shares several glycolipids with Mtb and that induced specific humoral immune responses against Mtb infection when it was enwrapped in liposomes[100].

5. Conclusions

Liposomes are essential drug delivery carriers characterized by a number of advantageous properties. They are generally able to safely carry therapeutic compounds to target cells, oftentimes increasing their therapeutic activity and preventing any toxic side effects. Their ability to induce specific immune responses serves as a fundamental feature of an effective delivery system and adjuvant in the development of TB vaccines. Nonetheless, further extensive research and development are required to optimize for a number of varying factors that determine the efficacy of liposomes in these applications, including the liposome sizes, surface charge, and composition of the lipid bilayers.

Conflicts of interest statement

We declare that there is no conflicts of interest.

Acknowledgements

The authors thank the Department of Immunology, School of Medical Sciences and School of Health Science, Universiti Sains Malaysia and funding agencies for supporting this study.

Funding

This study was supported by Fundamental Research Grant Scheme (FRGS/1/2018/SKK08/USM/03/1), Ministry of Higher Education (Malaysia) and Long-term Research Grant Scheme (LRGS/1/2015/USM/01/1/1), Ministry of Higher Education (Malaysia).

Authors' contributions

NEML, SA, ASNA and AN performed the literature search and drafted the manuscript. RK, VU, RM, MES, AA and MNNA supervised and revised the manuscript. All authors contributed significantly to the manuscript and approved the submitted version.

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Lambuk et al. Eye and Vision (2022) 9:26
https://doi.org/10.1186/s40662-022-00298-y

Eye and Vision

REVIEW Open Access

Nanoparticles for the treatment of glaucoma-associated neuroinflammation

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Abstract

Recently, a considerable amount of literature has emerged around the theme of neuroinflammation linked to neurodegeneration. Glaucoma is a neurodegenerative disease characterized by visual impairment. Understanding the complex neuroinflammatory processes underlying retinal ganglion cell loss has the potential to improve conventional therapeutic approaches in glaucoma. Due to the presence of multiple barriers that a systemically administered drug has to cross to reach the intraocular space, ocular drug delivery has always been a challenge. Nowadays, studies are focused on improving the current therapies for glaucoma by utilizing nanoparticles as the modes of drug transport across the ocular anatomical and physiological barriers. This review offers some important insights on the therapeutic advancements made in this direction, focusing on the use of nanoparticles loaded with anti-inflammatory and neuroprotective agents in the treatment of glaucoma. The prospect of these novel therapies is discussed in relation to the current therapies to alleviate inflammation in glaucoma, which are being reviewed as well, along with the detailed molecular and cellular mechanisms governing the onset and the progression of the disease.

Keywords: Nanoparticles, Ocular drug delivery, Neuroinflammation, Glaucoma, Retinal ganglion cell

Background

Glaucoma, a prime cause of irreversible blindness, refers to a group of ocular disorders with multifactorial etiology. As of now, it is considered a neurodegenerative disease in both the eye and brain [1]. In 2020, approximately 76 million people suffered from glaucoma and this number is expected to reach 112 million by 2040 [2]. These complex neurodegenerative disorders are characterized by optic neuropathy which is potentially progressive and visible changes can be seen at the optic nerve head (ONH) [3]. Glaucomatous optic neuropathy indicates a structural damage to the optic nerve with the corresponding loss of function. The structural damage is observed through the neurodegeneration of retinal ganglion cell (RGC) axons and deformation of lamina cribrosa with a concomitant diffuse and localized nerve fiber bundle pattern [4]. Undetected glaucoma in the early stages increases the risk of visual field loss [5]. The visual acuity may be spared at the early stage of the disorder, but progression of the neurodegenerative changes may result in the complete loss of vision [6].

Mechanisms underlying the development and progression of glaucoma at the level of RGC axons at the ONH remain unclear [7]. However, studies have indicated that early neuroinflammatory response is potentially a contributing factor to glaucomatous optic neuropathy (Fig. 1) [8]. Immunological surveillance in retina mediated by astrocytes, microglia, and other blood-derived immune cells is hypothesized to be associated with pro-inflammatory events leading to RGC damage [9, 10]. Several studies also found substantial evidence on the

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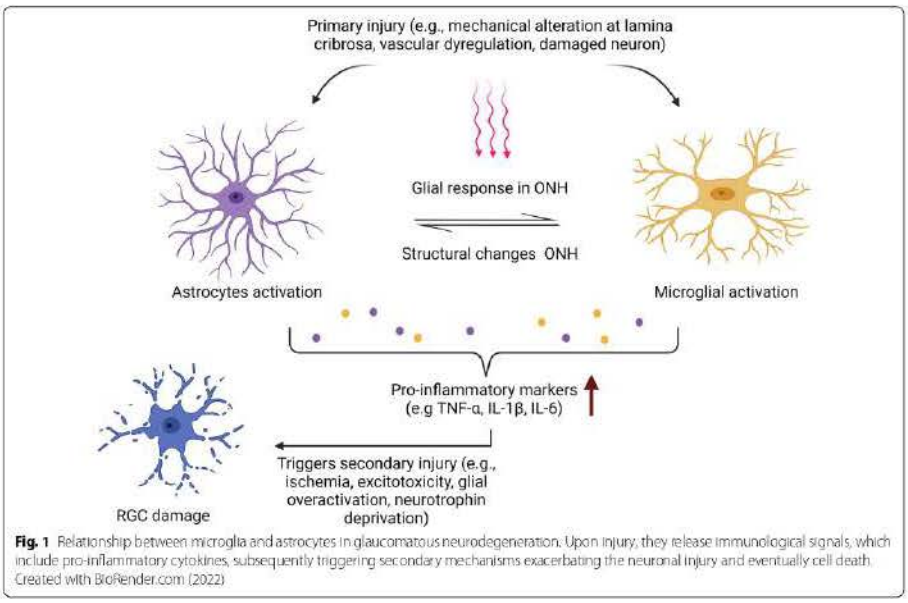

Fig. 1 Relationship between microglia and astrocytes in glaucomatous neurodegeneration. Upon injury, they release immunological signals, which include pro-inflammatory cytokines, subsequently triggering secondary mechanisms exacerbating the neuronal injury and eventually cell death. Created with BioRender.com (2022)

detrimental impact to axons, cell bodies, and dendrites of the ganglion cells during the early stage of experimental glaucoma in animal models [11, 12]. Interestingly, dampening certain pro-inflammatory pathways appears to have a neuroprotective effect on RGCs, particularly on events at the ONH during the early stages of glaucoma, further demonstrate the role of neuroinflammation in its pathogenesis [13]. Regardless of the initiation of insults, the neurodegeneration of ganglion cell axons is associated with the loss of ganglion cell bodies via apoptosis [14]. Since RGCs are unable to regenerate their axons, their loss is irreparable, which can disable the eye from generating connections to the brain and result in lifelong visual loss [14]. However, it calls for a greater concern because its prevalence is on the rise, and unlike in the case of cataracts, there is no effective therapy available [15].

The goal of any glaucoma treatment is to prevent vision loss. Most recent therapies have been focused on lowering intraocular pressure (IOP), as it is the only proven treatment for glaucoma; elevated IOP is considered the primary risk factor for the initiation and progression of the disease [3]. However, a significant number of glaucoma patients show worsening visual fields even when the IOP is controlled [16]. Although higher baseline IOP and older age are regarded as consistent and predisposing factors for glaucoma progression, several other factors should not be overlooked [17, 18]. In particular, individuals with family history of glaucoma, genetic predisposition, medications for pre-existing conditions such as systemic hypertension and diabetes, high myopia with great disc torsion of the optic disc and thinner lamina cribrosa at the ONH, and central corneal thickness, are amongst the factors reported that can influence the development of the disease [19, 20]. Nevertheless, to this day, IOP remains as the cardinal modifiable parameter in the management and treatment of glaucoma. In spite of that, most treatments for controlling IOP are associated with adverse effects and none of the current anti-glaucoma medications provide retinal neuroprotection by preventing RGC loss [21]. Although more than a few emerging therapeutic agents seem to have the potential to provide neuroprotection in human glaucoma, none of them have been clinically approved so far. Thus, there remains a need for therapeutic interventions that can provide maximal retinal neuroprotective effects in glaucoma with minimal adverse effects.

Glaucoma drug therapy typically employs topical instillations of eye drops. Although other glaucoma treatments such as surgical and laser therapy are increasingly

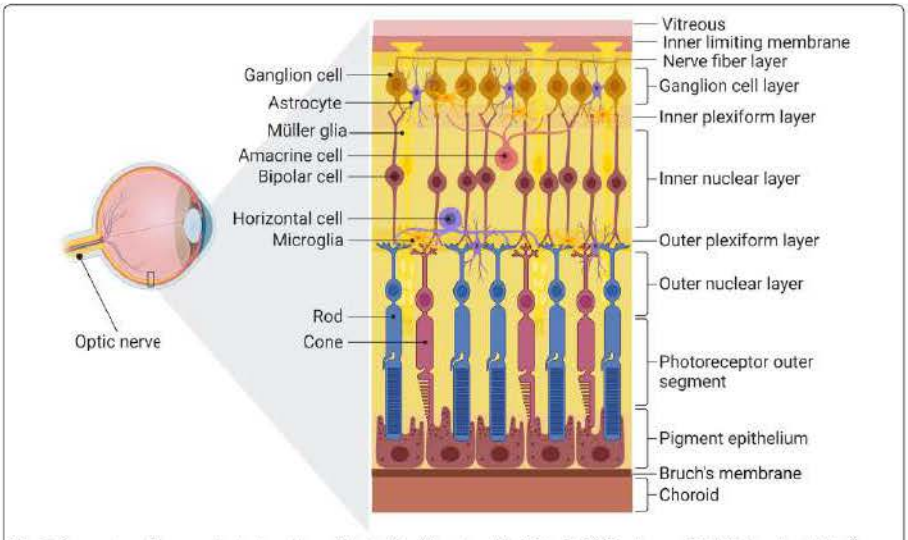

Fig. 2 The overview of human retinal cells and layers (Adapted from "Structure of The Retina", by BioRender.com (2022). Retrieved and edited from https://app.biorender.com/biorender-templates)

utilized in the clinical setting, conventional eye drop remains the primary treatment for the majority of glaucoma cases [21]. Owing to various anatomical and physiological barriers in the eye, it is highly challenging for the drug to reach the target site [22]. Topical application of the glaucoma drug is predicted to reach the target tissue at the amount not higher than 5% of the applied amount due to the rapid clearance mechanisms at the corneal surface [23]. In addition, poor instillation by patients, especially the elderly, and the drug overspill are other contributing factors to the low bioavailability of ocular drugs in glaucoma [24]. To circumvent these obstacles, a new paradigm for glaucoma medical therapy is needed to fulfil the gold standard of the treatment criteria, including the efficient reduction of the IOP in such a way that the visual field is not compromised, and the optic nerve protected without causing tachyphylaxis and without generating other local and systemic adverse effects. It is also important to consider a treatment that can promote patient compliance and applicability in diverse patient populations [25].

Among many new therapeutic innovations for the treatment of glaucoma, nanoparticles (NPs) occupy a prominent place [26]. In this review, we have examined the potential of NPs in the treatment of glaucoma by emphasizing the mitigation of neuroinflammation, all to circumvent the drawbacks in the current glaucoma therapies.

Main text

Modulation of neuroinflammation in glaucoma

Neuroinflammation in glaucoma can take place at different physiological locations, but it is most prominent at the posterior segment of the eye (i.e., retina and optic nerve; Fig. 2) and the brain (i.e., superior colliculus and lateral geniculate). It can also occur peripherally in blood vessels. Nonetheless, the primary focus has been on the RGCs. In the ONH, most research has demonstrated the critical concern of glaucoma in which the RGC soma, synapses and dendrites show the effects of neuroinflammation and peripheral immune responses. Recent studies have demonstrated leukocytic recruitment into the ONH and the retina, which may contribute to the development of the glaucoma [13, 27].

In the pathophysiology of glaucoma, RGC axons are the first to be affected. Mechanical alterations to lamina cribrosa, neurotrophic signaling, direct RGC pressure, and neuroglial activation, such as that of microglia or astrocytes, are among the initial stimuli for this event. Müller glia, astrocytes, and microglia are the 'resident

cells' that stimulate innate immune responses in the retina and optic nerve. The astrocytes play a crucial role in controlling homeostatic conditions for neurons by maintaining neurovascular coupling (neuronal activity/local blood flow) [28, 29]. When activated, astrocytes undergo morphological alteration and proliferation to the area of injury. Severe astrocytosis, an abnormal increase in the number of astrocytes that leads to inflammatory responses, has been reported in glaucoma and is known to be involved in the onset of the disease [30]. Some studies have reported that ONH astrocytes have a phagocytotic effect which are able to engulf synaptic materials and cellular debris [31, 32]. Nevertheless, the extent of effects of astrocytosis is still the subject of ongoing research.

In glaucomatous pathophysiology, microglia and macroglia are the key players responsible for immunoregulation in the retina [33]. These cells are responsible in several key functions including providing nutritional and structural support, regulating metabolic activity and homeostasis, phagocytosis as well as levels of cytokines and neurotrophic factors [34].

Major neuroinflammatory cells in glaucoma

Microglia is the key cell type controlling neuronal function and homeostasis. They are phagocytes that play a vital role in the innate immune response. As resident macrophages, its presence is undoubtedly ubiquitous in the central nervous system (CNS) [35]. This cell is the first to react toward the site of injury by stimulating inflammatory cascades and recruiting other inflammatory cells, such as astrocytes. In an in vivo study of glaucoma, activated microglia were found to increase in number in glaucoma; however, it is not certain whether these reactions are beneficial [36]. Initially, microglia were assumed to have ascended from the yolk sac of macrophages that had entered the brain during the development of fetus. However, more recently, it is believed to have come from circulated monocytes, which later differentiated into microglia [37]. Microglia are responsible for homeostasis of the neural circuits and angiogenesis in the retinal development. In mature retina, microglia help in neuronal signaling and integrity of synaptic transmission [38–40]. Some studies have suggested that inactivation of retinal and ONH microglia using drugs known as minocycline lowers the neurodegenerative actions [41, 42]. Astrocytes, microglia, and macrophages are believed to be involved in neuronal inflammation, with aging among the causative factors [43].

In glaucomatous eyes, activation of microglia has been detected at an earlier stage, whereas the aggregation, activation and redistribution of microglia is seen even before RGC injury has taken place in a DBA/2J mouse model of chronic hereditary glaucoma [44]. The early phase of the glaucomatous model in mice also involved the monocytic recruitment and other pathologies with neuronal damage [45]. Microglial activation and proliferation can lead to detrimental effects on RGCs through the secretion of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and reactive oxygen species (ROS) [46]. In another study utilizing the same animal model, the transcriptome of ONH microglia was seen to change drastically in the metabolic, phagocytotic, inflammatory and sensome pathways [47]. This was confirmed by another study, which showed an increased activity of microglia and their density in the retina, including the optic nerve when IOP was elevated [36]. This finding supports the hypothesis that the chronic ocular hypertension inhibits the homeostasis-regulating function of microglia [47]. Despite all these findings, the role of microglia is still debatable. Few studies have suggested that RGC injury can be worsened by microglia when the inflammatory mediators such as TNF-α, interleukin 1β (IL-1β), IL-6, matrix metalloproteinases, Fas ligands (FasL), and ROS are released [46, 48].

Macroglia are predominantly Müller cells and astrocytes that share similar transcriptomic profiles and functions [49]. Out of these two cell types, the prime macroglial cells are Müller cells, which can be found across the retina. Their cell bodies lie in the inner nuclear layer, which elongates into two trunks that extend their ends into the inner limiting membrane. The inner limiting membrane separates the retina from the vitreous body and is one of the most significant barriers for ocular drug delivery [50]. For a detailed overview of the inner limiting membrane, the reader is referred to the work by Peynshaert et al. [50–52]. Müller cells are essential in maintaining the structural integrity of the retina. They are also important regulators for cell metabolism in the retina [53]. Müller cells are anatomical conduits among the retinal neurons, including the cellular environment. Hence, they help in maintaining retinal homeostasis. Astrocytes are the foremost glial cells found at the ONH. As the dominant component of glial cells in the CNS, astrocytes engage in a variety of critical functions, such as ionic balance regulation, metabolic supply and its structural maintenance, neurotransmitter transmission, and synaptic plasticity [54]. Collectively and together with microglia, astrocytes and Müller cells ensure a smooth process for the synaptic activity to occur by maintaining ion and neurotransmitter levels.

When an injury occurs to the retina, macroglia can be stimulated to release glial fibrillary acidic protein (GFAP) and other extracellular matrix proteins [55, 56]. In the glaucomatous retina, there is an increased amount of GFAP immunostaining and macroglia display

a hypertrophic morphology, suggesting the existence of retinal gliosis in glaucoma [57]. Several models of glaucoma have showed that the number of astrocytes increases with increased GFAP immunoreactivity, and this was also seen on the extracellular matrix remodeling on the ONH. GFAP is an intermediate filament of the glial cell cytoskeleton that upsurges when astrocytes transform into their reactive state [55]. Furthermore, some studies have shown that ONH astrocytes play a significant role in the engulfment of optic axons, including stimulation of axon degradation, which is one of the proposed mechanisms for the sectorial nature of RGC loss in glaucoma [32]. The inflammatory response in glaucoma is activated and mediated early on through a process called astrogliosis. Several inflammatory pathways were activated by astrocytes when rats were injected with hypertonic saline into their episcleral veins, leading to a high IOP [58]. The activation of inflammatory pathways includes tumor necrosis factor α (TNF-α) signaling, nuclear factor kappa B (NF-κB) activation, autophagy, and inflammasome-associated regulators.

Inflammatory pathways

TNF-α and toll-like receptors (TLRs) pathways are among the crucial complement cascades in glaucomatous neuroinflammation. Furthermore, there are various associated inflammatory mediators involved such as cytokines and prostaglandins [59], and pathways such as β2-microglobulin and cluster of differentiation 3 (CD3) [60]. Studies are currently focused on the classical pathway of the complement cascade whereby RGCs detect stimuli of injuries and activates the complement component 1 (C1) complex, a giant proteolytic enzyme [61–63]. This is followed by activation of the C3 convertase, which can attract leukocytes and further activates C5 convertase. C5 convertase would recruit more leukocytes and stimulate cell lysis through the membrane attack complex. In the complement cascade in a glaucomatous eye, glial cells such as astrocytes would amplify the RGC signal to boost microglia response and even attract monocytes, especially in the ONH and inner plexiform layer. Rather than killing the RGCs, the complement system is believed to protect them from further damage and maintain their function [64]. Still, based on the previous glaucomatous animal model, the nature of the neuroinflammatory mechanism is thought to possess an evidently damaging role on the disease [65, 66].

Meanwhile, TLRs pathway induce glaucomatous neuroinflammation in two ways, either through polymorphism of TLR4 alleles [67] or increase in TLR4 protein expression in the retina of glaucoma animal models [68]. Different TLRs recognize different stimuli. For instance, TLR3 detects double-stranded RNA of foreign substances, whereas TLR4 focused on detecting an endogenous ligand, for example, tenascin-C, which is elevated in the glaucomatous ONH [69, 70]. It has also been found that monocytes and microglia have more detecting TLRs as compared to astrocytes. Even though some studies have examined TLRs in RGC injury, more investigation is needed to further delineate the role of TLRs in human glaucoma.

Another critical activator of neuroinflammation in glaucoma is TNF-α, which is produced by astrocytes and especially microglia [71, 72]. Studies have reported that polymorphism [73, 74] and increment of TNF-α in the vitreous body, retina and optic nerve are associated with glaucoma [75, 76]. Parallel to FasL's downstream actions, TNF-α also triggers RGC cell death [77, 78]. In a vitreous glaucoma mouse model, oligodendrocyte and RGC damages are induced by soluble murine TNF-α while TNF-α suppression prevented these damages [43, 79]. This is similar to the extent of blocking FasL activity by pharmacotherapy [80]. Although TNF-α inhibitors seem to have a neuroprotective property in clinical settings, further research is needed to clarify this, especially in glaucomatous diseases [74].

Several pathways of neuroinflammation have been proposed, although more extensive studies are required for both in vivo and human glaucoma. The continued elucidation of these pathways is essential for defining therapeutic targets that have clinical benefit.

Current investigational therapies to alleviate inflammation in glaucoma

Immunomodulatory drugs

With respect to the neurodegenerative potential of neuroinflammation, several molecules have showed the potential to act as 'neuroprotectors'. Citicoline (cytidine 5′-diphosphocholine) exemplifies a naturally endogenous compound that has been evaluated for its protective role on RGC in glaucoma [81]. A number of in vitro and in vivo studies have demonstrated the neuroprotective role of citicoline via increased dopamine retinal levels, enhanced anti-apoptotic effect, restrained thinning of the retinal nerve fiber layer (RNFL), regeneration of neurites, defense against glutamate excitotoxicity, and minimized RGC impairment, thereby enhancing a better visual field [82]. A significant reduction in the apoptotic nuclei pathway of cell death with contrasting synaptic loss achieved with citicoline treatment showed its efficacy in protecting against the excitotoxic neuronal damage and thus delayed the progression of glaucoma [83, 84]. Citicoline also plays a crucial role in the regeneration of the axon through sphingomyelin synthesis, which stabilizes the plasma membrane of RCG axons, thereby suppressing free fatty acids and protecting against the redox imbalance [85].

Experimental studies in adult male Albino rabbits treated with citicoline had demonstrated a higher dopaminergic neurotransmission in the brain compared to the untreated group, and highlighted the influence of citicoline on retinal catecholamine levels [86]. The usage of citicoline against retinal damage eventually proved to have a neuroprotective effect in kainic acid-induced neurotoxicity in vivo [87]. Shuettauf et al. investigated the antiapoptotic effect of mitochondria-dependent cell death mechanism by delivering citicoline with lithium, with the outcome being a rise in the RGC density [88]. In a randomized clinical trial, Parisi et al. observed, enhanced retinal and visual functions in a glaucoma patient who received citicoline [89]. A follow-up electrophysiological analysis of glaucomatous visual dysfunction, which was carried out in conjunction with hypotensive therapy, further confirmed citicoline to be a fitting medical treatment for glaucoma within an extended period of time [90]. The effects of citicoline administered through oral and intramuscular approaches were subsequently tested on glaucoma patients with moderate visual defects, showing improvement in retinal function [91]. In a similar study conducted by Ottobelli et al. patients with progressing glaucoma were supplemented with an oral citicoline solution and the follow-up visual examinations showed reduced rate of mean progression by the end of the study after the treatment [92]. Lanza et al. demonstrated neuroprotective effect of oral citicoline, which slowed down the progression of primary open-angle glaucoma (POAG). The citicoline therapy assessment by standard automated white-on-white perimetry showed a stable and highly significant mean deviation (MD) of progression over time in treated patients compared with untreated patients [93]. Another method carried out in a different study acknowledged that intravenous therapy can be a means for citicoline to reduce the progression of glaucoma in conjunction with citicoline eye drops as the IOP lowering treatment. Visual field and RNFL loss detected were much lower on average [94]. Studies involving the encapsulation of citicoline eye drops in a liposomal formulation conducted by Parisi et al. also suggested improved retinal bioelectrical responses with enhanced visual cortex bioelectricity [95]. Overall, these findings showed the crucial role of citicoline as a neuroprotective compound for managing glaucoma, yet further clinical trials with larger sample sizes are highly needed to gather more understanding in relation to the dose-response and clinical effects.

The renin-angiotensin aldosterone system (RAAS) is a complex endocrine system that has a major function in the regulation of hemodynamic stability and fluid balancing. Upon the occurrence of hypotension in the body, granular cells of renal juxtaglomerular apparatus release the renin enzyme, which cleaves angiotensinogen to angiotensin II (Ang II) via Ang II type 1 receptor (AT1-R) [96]. Recent evidence suggests the prospect of utilization of AT1-R antagonists as a treatment for several conditions such as hypertension, blood pressure and cardiovascular diseases, mainly due to the pro-inflammatory effects of Ang II and aldosterone [97]. Studies have proven that administration of AT1-R blockers is not only able to transverse the blood-brain barrier and communicate with AT1-R to minimize the infarct volume, but also extenuate inflammatory and oxidative stress in the retina and brain [98]. Yang et al. showed that the AT1-R signaling blockade of candesartan succeeded in averting the retinal neuronal death in a rat model of chronic glaucoma [99]. Similarly, the orally active AT1-R antagonist candesartan inhibited toll-like receptor 4 (TLR4-apoptosis signal-regulating kinase 1 pathway), which supported the activation of RAAS in the innate immune response, expediting neural cell death [96]. The conclusion is a significant neuroprotective effect of Ang II against RGC loss.

Natural products

Aside from the standard therapy used currently, which involves IOP reduction through medical drugs, laser and surgical therapy, herbal medicine is one of the primary alternatives chosen in the management of glaucoma [100]. In the nineteenth century, active compounds were directly isolated from plants [101]. Plants such as ginkgo biloba, saffron, and phytochemicals such as epigallocatechin-3-gallate and resveratrol are known as traditional remedies used in glaucoma pathology [102].

Among various antioxidative compounds present, Ginkgo (Ginkgo biloba), which originated from China 250 million years ago, has been recognized for its therapeutic effects in several pathologies, including neurodegenerative diseases [103]. The beneficial component of this living fossil tree is found in the ginkgo extract, which contains polyphenolic flavonoids that stabilize the mitochondria at organelle level, and also exerts multiple therapeutic properties, including the antioxidant, antimicrobial, neuroprotective and antiapoptotic effects [104, 105]. Extract 761 (EGb761), obtained from leaves of the ginkgo plant, has been effective in treating Alzheimer's dementia and cognitive impairment. Therefore, researchers attempted to use EGb761 in the treatment of glaucoma due to the analogous biological and mechanistic features between these two chronic disorders [106]. Namely, both Alzheimer's dementia and glaucoma are age-related pathologies, experiencing the RGC degeneration and deposition of extracellular fibrils in the exfoliation syndrome, indicating that both are likely derived from similar misfolding mechanisms [107]. In previous studies, both short- and long-term

effects of the ginkgo biloba extract (GBE) were tested and the extract was used to treat pre-existing patients with normal tension glaucoma (NTG), often resulting in a significant improvement of visual acuity [108, 109]. However, Guo et al. who performed a randomized, crossover clinical trial, failed to demonstrate the effect of GBE to improve progressing visual defects within normal NTG patients, likely due to the smaller sample size and shorter time periods applied during the study [110]. The administration of GBE also showed an increasing end diastolic velocity in the ophthalmic artery and NTG throughout clinical cross-over trials, highlighting the desirable effect of the drug on the retinal blood flow in glaucoma disorders [111, 112]. Shim et al. supported these findings by showing the escalating MD upon GBE and bilberry anthocyanin treatment [113]. In a similar study, the standardized EGb761 extract demonstrated a progressing pharmacological effect on the oxidative stress with improved vascular circulation in both in vitro and in vivo experiments, highlighting the neuroprotective effect of the drug against the hypoxic injury of RGCs [114]. These findings have emphasized the prospect of this natural medicine in treating glaucoma. However, its usage has yet to become widely recognized in public.

Saffron, the dried stigmas originating from the *Crocus sativus* flower of the Iridaceae family in Greece, has been commonly used in cooking as an aromatizing and coloring seasoning [115]. The major constituents in saffron are natural carotenoid compounds, namely crocin and crocetin [116]. Its usage in the medical field has been recognized in the treatment of various diseases due to the wide therapeutic spectrum, including neuroprotective, anti-inflammatory, anti-oxidant and anti-genotoxic activity [117]. Both saffron compound extracts, crocin and crocetin, showed an enhanced neuroprotective effect through repression of activated microglia neurotoxicity. The development of intracellular ROS and nitric oxide is inhibited with a slower release of TNF-α and IL-1β [118]. These beneficial aspects can be observed in animal models of neurodegenerative ocular diseases and patients suffering from diabetic retinopathy and age-related macular degeneration (AMD). Studies in animal models with retinal damage emphasized the role of crocin in saffron as an inhibitor of the ischemic damage and a stabilizer of the ocular blood flow, alongside the neuroprotective effect provided by crocetin [119]. In a pilot study, Bonyadi et al. investigated the influence of an aqueous saffron extract on the IOP in the eyes of POAG patients and showed that the treatment significantly decreased the mean baseline IOP compared to the control group by the end of the therapy [120]. Despite the limited studies on saffron in glaucoma disease, the saffron extract emerges as an important therapeutic agent for potential clinical use.

Epigallocatechin-gallate (EGCG) is a type of catechin mainly found in green tea. It is well known as a robust antioxidant with multifunctional properties and has been investigated for its contribution to neuroprotection in human corneal epithelial cell culture models and animal models of glaucoma [121, 122]. Earlier findings not only demonstrated its therapeutic effect on the axon and the bodies of RGCs in optic nerve crush and *N*-methyl-D-aspartate (NMDA) toxicity studies, but also showed an elevation in the survival rates of RGCs via oral administration [123, 124]. In a similar study, which also used oral EGCG, the drug was shown to be a potent penetrator into the retina, where it reduced both the injury caused by ischemia and in vitro white light-induced apoptosis in RGC-5 cells [125]. Falsini et al. claimed a higher amplitude detection in the OAG group compared to the ocular hypertension (OHT) group in a pattern electroretinogram analysis, thus supporting the prospect of short-term supplementation of EGCG [121]. ECGC does not only provide protection against the oxidative stress, but also has the capability to weaken the glutamate-induced cytotoxicity by decreasing the ionotropic calcium influx [126]. These outcomes showed that EGCG is a suitable neuroprotective agent for the glaucoma treatment. However, there is a need to perform further studies to determine the long-term benefits, the component activity, and the precise dosage requirements for EGCG in the glaucoma treatment.

Resveratrol (RSV), also known as 3,5,4′-trihydrocystilbene, a nonflavonoid polyphenol compound derived from plant sources such as grapes, blueberries and apples, has been developed into an effective phytoalexin [127]. It has diverse roles in relation to the well-being of humans, by virtue of biological attributes including antioxidant, anti-inflammatory and neuroprotective functions [128]. In POAG patients, RSV was shown to interrupt intracellular ROS, inhibit the release of inflammatory cytokines and slow down the accretion of carbonylated proteins, hence supporting the neuroprotective action of the drug against the RGC apoptosis and the ability to slow down the progression of glaucoma [129]. Other studies also demonstrated this neuroprotective effect of RSV, including the delay in the RGC loss upon dosing with RSV and riluzole. Although both single and combined administrations were effective, an improved and better RGC protection was provided through the combined therapy [130]. Luo et al. showed that sirtuin 1 (SIRT1) activation by RSV confers neuroprotection in mice with ischemia–reperfusion injury (IR) via Akt activation and mitochondrial apoptotic suppression with a verified concentration of

intravitreal injection, and thus contributed to the understanding of the mechanism of action important for the clinical usage of RSV [131]. Inhibition of endothelin-1, a vasoactive peptide in glaucoma, highlighted a pivotal effect of RSV [132]. Moreover, researchers have suggested the induction of mitochondrial biogenesis by RSV to alleviate glaucomatous retinopathy. This is due to the efficiency of RSV in reducing derivative-serum in the RGC-5 cell line by subcellular translocation of SIRT1 dependent proliferator-activated receptor-gamma coactivator 1 alpha [133]. In addition, Shamsher et al. studied the in vitro and in vivo neuroprotective effects of RSV and curcumin nanoparticle formulations with ~70% encapsulation efficiency [134].

One of the examples of long-standing, well-conducted research and development of an anti-inflammatory agent comes from curcumin, a major active compound of turmeric, Curcuma longa [135]. Curcumin has shown exceptional promise for the beneficial modulation of numerous signaling molecules (e.g., pro-inflammatory cytokines, NF-κB, apoptotic proteins, and C-reactive protein) in multiple diseases, including cancers and inflammatory and neurodegenerative disorders. Apart from anti-inflammatory properties, curcumin exerts antioxidant, anti-microbial and anti-tumorigenic activity. Owing to these properties, curcumin has been extensively studied in vitro and in vivo in the context of many inflammatory, autoimmune, and degenerative diseases of both anterior and posterior segment, and has been suggested as an adjuvant therapy [136]. In a retinal ischemic injury animal model, curcumin was reported to prevent ischemic damage to the RGC and microvasculature via suppression of NF-κB signal transducer as well as activation of transcription 3, and monocyte chemotactic protein 1 expression [137]. The chemical properties in curcumin with anti-inflammatory and antioxidant functions have been suggested to be associated to its hydroxyl and methoxy group, which deregulates TNF-α and pro-inflammatory interleukins which lead to the downregulation of STAT pathways. In both in vitro and in vivo experimental glaucoma studies, curcumin has shown antioxidant effects, as demonstrated by the improved cell viability of microglial cells, reduced intracellular ROS and apoptosis of RGCs [138]. These findings should make an important contribution to the therapeutic potential of curcumin in clinical ophthalmology, notwithstanding that this potential is restricted by a few adverse factors, including extremely poor bioavailability and water solubility [139]. The active fraction of curcumin detected in the blood is often suboptimal, for which reason increased doses are needed to achieve the proper therapeutic effect [140]. To overcome these limitations, several approaches such as the use of enhancers, analogues and nanocarriers to provide a hydrophobic environment for poorly water-soluble curcumin have been reported and extensively reviewed by other [141–143]. An in vivo study by Davis et al. demonstrated < 95% encapsulation efficiency with good stability upon the formulation of topical curcumin-loaded, Pluronic-F127 stabilized D-α-tocopherol polyethene glycol 1000 succinate NPs (< 20 nm) [143]. Similarly, Cheng et al. developed a formulation consisting curcumin-latanoprost NPs (~ 161 nm), which resulted in a sustained-release profile with low oxidative stress-mediated damage via ROS production and apoptosis in vitro and in vivo [144]. These studies highlight the potential of curcumin to provide a neuroprotective therapy in glaucoma. Of note, the use of nanocarriers is one of the most prospective approaches in improving curcumin delivery. With the development of a nanocarrier suitable for utilization as a topical formulation, the bioavailability of curcumin could be drastically improved [143]. One of the promising drug carriers for the delivery of curcumin has been the amphiphilic polymer polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer, Soluplus [145]. Although this and other types of carriers must be further investigated, they provide important opportunities for advancing the understanding of curcumin as an anti-inflammatory agent and, potentially, as a neuroprotective therapy in glaucoma.

The above mentioned substances were extensively studied in both IOP-dependent and -independent types of glaucoma. Outside traditional clinical settings, the progression of glaucoma can be controlled, yet it still cannot substitute the conventional therapeutic management of glaucoma; hence, further studies are required. It is noteworthy that an increasing number of experimental studies currently consider molecular targets in the modulation of inflammatory responses activated by microglial cells, RGCs and other retinal cells that elicit downstream actions of inflammatory pathways responsible for glaucomatous neurodegeneration (Table 1). Although the therapeutic anti-inflammatory potential of the various agents seems to be encouraging, their neuroprotective effects could be attributed to other factors, including the route of delivery to the target tissues, which may have an impact on patient safety and compliance in clinical practice [3]. Most of the therapeutic agents also require a proper formulation to provide optimal neuroprotection, particularly in promoting RGC survival under glaucomatous conditions. To fully explore the potential of these therapies and their biocompatibility for the treatment of glaucoma in humans, further investigations are necessary to develop formulations that can be administered non-invasively.

Table 1 Recent therapeutic options on anti-inflammatory and neuroprotective effects in experimental models of glaucoma and other ocular disease-associated RGC loss

Therapeutic agent	Experimental model	Route of delivery	Anti-inflammatory and neuroprotective effects	Refs.
Magnesium acetyltaurate (MgAT)	Retinal ischemia injury; retinal excitotoxicity injury in rat	Intravitreal injection	• Suppressed ET-1- and NMDA-induced retinal and optic nerve damage through induction of iNOS, suppression of NF-κB p65, p53, AP-1 (c-Jun/c-Fos) signaling pathways, downregulation of TNF-α, IL-1β, IL-6, and caspase-3 • Preserved RGC survival by ~ten-fold in NMDA-induced group • Improved visual function after 7 or 14 days of treatment	[217, 218]
Dietary supplementation (combination of forskolin, homotaurine, spearmint, and B vitamins)	IOP elevation in mice	Oral	• Maintained IOP at baseline level 2 weeks before and after supplementation • Suppressed elevated IOP-induced NF-κB signaling pathway and reduced caspase-3 activity • Preserved retinal function and 20% RGC survival more than the untreated group	[219]
Laquinimod (LQ)	Retinal ischemia and reperfusion injury in mice	Topical	• Reduced numbers of activated microglia • Suppressed retinal TNF-α, IL-1β, IL-6, and iNOS levels • Inhibited caspase 8 and NLRP3 in retinae and microglia • Promoted RGC survival ~ 1.9-fold and preserved retinal function	[220]
ONL1204 (small peptide Fas antagonist)	IOP elevation in mice	Intravitreal injection	• Abrogated microglial activation by ~ 1.9-fold • Downregulated cytokines and chemokines, macrophage inflammatory protein (MIP), MIP-1α, MIP-1β, MIP-2, monocyte chemoattractant protein-1 (MCP1), interferon gamma-induced protein 10 (IP10), TNF-α, IL-1β, IL-6, and IL-18), caspase-8, components of the complement cascade (C3 and C1Q), TLR4, and NLRP3 • Prevented axon degeneration ($P < 0.0001$) and preserved RGC survival ($P < 0.001$) • No significant different in IOP	[221]
Apolipoprotein E (ApoE)-mimic peptide COG1410	Optic nerve crush injury in mice	Intravenous injection	• Reduced JNK phosphorylation, TNF-α, IL-1β, IL-6, iNOS, and Bax/Bcl-2 ratio • Promoted RGC survival by ~ 61% and reduced optic nerve damage ($P < 0.05$) • Preserved visual function	[222]
Caffeic acid phenethyl ester	Optic nerve crush injury in rat	Intraperitoneal injection	• Downregulated retinal glia-mediated NF-κB activation, IL-8, IL-6, iNOS, COX-2, and TNF-α • Attenuated gliosis ($P < 0.01$) • Enhanced RGC survival ($P < 0.001$)	[223]

Table 1 (continued)

Therapeutic agent	Experimental model	Route of delivery	Anti-inflammatory and neuroprotective effects	Refs.
Green tea extract (Theaphenon E)	Retinal ischemia and reperfusion injury in rat	Intragastric administration	• Downregulated TLR4, TNF-α, and IL-1β levels • Reduced expression of cleaved Caspase-3 and Caspase-8 • Downregulated expression Superoxide dismutase 2 (SOD-2), Janus kinase (Jak) and p38 • Enhanced RGC survival ($P < 0.001$) in ischemic retina	[224]
Kaempferol	Retinal ischemia and reperfusion injury in mice	Intragastric administration	• Downregulated expression levels of TLR4, TNF-α, IL-1β and IL-6 • Inhibited activation of NF-kB and JNK signaling pathways • Reduced active caspase-3 and caspase-8 • Prevented NLRP1/NLRP3 inflammasome activity • Prevented IOP-induced RGC death ($P < 0.01$)	[225]
Minocycline	Retinal vein occlusion in rat; retinal ischemia–reperfusion injury in mice	Intravenous injection	• Reduced activation of microglia • Reduced RGC loss (~ 45%, $P < 0.05$) • Improved visual function	[226]
Omega-3 polyunsaturated fatty acids	Anterior ischemic optic nerve injury in rat	Oral gavage	• Downregulated TNF-α, IL-1β, and iNOS levels • Reduced macrophage polarization • Survival of RGC in central and midperipheral retinas was ~ 2.3—($P = 0.03$) and 2.0-fold ($P = 0.03$) higher • Reduced postinfarct apoptosis of RGCs by ~ 2.9-fold ($P = 0.007$)	[227]
Synthetic sterol (HE3286)	IOP elevation in rat	Oral gavage	• Maintained IOP at baseline level ($P = 0.997$) after oral delivery • Increased brain-derived neurotrophic factor (BDNF) expression and reduced TNF-α expression in the ONH • Reduced retinal IL-6, IL-1β, and p75 expression levels • Reduced microglia activation and reduced NF-kB localization • Increased NF-kB localization to neuronal nuclei in the superior colliculus and retina	[228]
4-(Phenylsulfanyl)butan-2-one	Optic nerve crush in rat	Subcutaneous injection	• Inhibited iNOS/COX-2 pathway in microglia • Increased RGC survival by ~ 36% in the central retina and ~ 35% in the mid-peripheral retina • Reduced RGC apoptosis by ~ 2.2-fold • Preserved visual function • No data on IOP comparison	[229]
Caffeine	Ocular hypertension in rat	Oral	• Partially reduced IOP level ~ 1.3-fold ($P < 0.001$) • Inhibited OHT-induced microglial activation • Reduced retinal TNF-α, IL-1β, and iNOS expression levels • Preserved RGC loss by ~ 1.8-fold ($P < 0.05$) but not RGC retrograde transport	[65]

Table 1 (continued)

Therapeutic agent	Experimental model	Route of delivery	Anti-inflammatory and neuroprotective effects	Refs.
Granulocyte colony-stimulating factor (G-CSF)	Optic nerve crush injury in rat	Subcutaneous injection	• Suppressed microglia activity • Downregulated TNF-α, IL-1β and iNOS expressions • Protected RGC from secondary degeneration injury by ~ 38% ($P < 0.01$) • No data on IOP comparison	[230]

AP-1 = activator protein 1; *Bax* = bcl-2 associated x; *Bcl-2* = b-cell lymphoma-2; *C1* = complement component 1; *C1Q* = complement component 1Q; *COX-2* = cyclooxygenase-2; *ET-1* = endothelin-1; *IOP* = intraocular pressure; IL = interleukin; *iNOS* = inducible nitric oxide synthase; *JNK* = c-Jun N-terminal kinase; *NF-κB* = nuclear factor kappa B; *NLRP* = NOD-, LRR-family pyrin domain; *NMDA* = *N*-methyl-D-aspartate; *MCP* = monocyte chemotactic protein; *MIP* = macrophage inflammatory protein; *RGC* = retinal ganglion cell; *TNF* = tumor necrosis factor; *TLR* = Toll-like receptor; *OHT* = ocular hypertension; *ONH* = optic nerve head

Potential of nanoparticles in drug delivery

Current perspective on glaucoma therapies targeting neuroprotective agents

Neuroprotection in glaucoma refers to any IOP-independent intervention that preserves the optic nerve by preventing or delaying RGC and axonal degeneration [146]. Regardless of the various definitions, neuroprotection is a therapeutic approach directed at keeping RGCs alive and functional in progressive glaucomatous optic neuropathy [147]. Data from randomized controlled clinical trials show that even with excellent IOP control the disease is still exacerbated in some patients [148]. Therefore, the idea of IOP-independent treatment strategies in glaucoma should be extensively investigated.

There is a vast amount of literature on identifying neuroprotective agents targeting the mechanisms proposed that underlie RGC damage in glaucoma [149]. Glutamate excitotoxicity antagonists, neurotrophic factors, and oxidative stress suppression are some of the studied neuroprotective agents with favorable neuroprotective activities. For further details regarding these, the reader is referred to the comprehensive reviews by Sharif and others [3, 150–153]. However, results from human clinical trials have been inconclusive and non-consequential [154]. Over time, literature reports have shifted towards inflammatory and immune responses, supporting the notion that neuroinflammation could be the key player in the mechanism underlying retinal damage in glaucoma, potentially having a reciprocal causative role in the pathology [69]. Since the mediators of neuroinflammation activate the immune system within the CNS, they may have either harmful or beneficial effects on RGC survival. This justifies a dire need for better therapeutic strategies. As such, the development of new therapies aimed at modulating rather than suppressing neuroinflammation might also produce the highly sought-after neuroprotective effects.

Challenges in ocular drug delivery

The outcomes of clinical trials testing for the safety and efficacy of neuroprotective agents demonstrate clear challenges in the aspect of drug delivery [146]. Poor drug delivery could be a factor largely contributing to the failure of the drug in clinical studies. The most common administration routes for glaucoma drug delivery are intravenous, subcutaneous, topical, and oral [155]. However, through these routes, ocular drugs are prone to absorption into the systemic circulation, which may result in low dose delivery to the target tissue and increase systemic risks. The low dose delivery could be also due to the poor solubility of the drug entailing high degradation rate and the failure to pass through the cornea and across the blood-retina barrier [156]. For topical administration, the corneal and the non-corneal routes control and influence the absorption [22]. The course that the drug molecules take towards the target cell in the intraocular environment starts with their passive diffusion via barriers formed by tight interconnected junctions. The components of these barriers include the precorneal pocket, corneal epithelium, the blood aqueous barrier, the retinal pigment epithelium and the blood capillary endothelial cells (choroidal barrier), all of which inevitably restrict the permeation of drug molecules into the intraocular chamber where they are to carry out their pharmacological action, resulting in an inefficient therapy [157]. Even if it were perfectly efficient, this trajectory would hardly allow for the access of the drug to the posterior area of the eye, where RGCs and the optic nerve reside. The most common route of administration to treat this posterior segment of the eye in experimental studies has been through intravitreal injection [158]; this is an immediate and direct route, which can increase the therapeutic drug delivery to the vitreous cavity and bypass the aforementioned barriers [159]. The intravitreal route is safe and effective, but due the invasiveness of the procedure, it is accompanied by side effects such

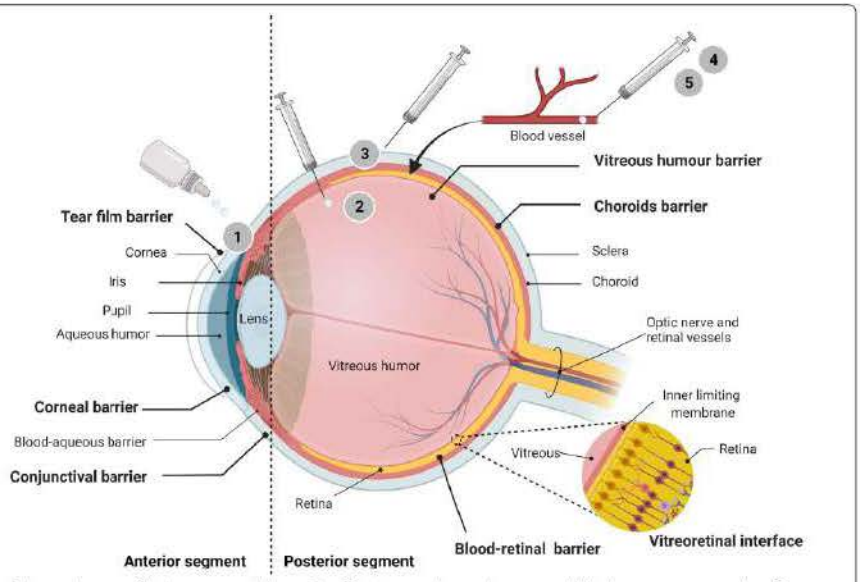

Fig. 3 Schematic diagram of the human eye with barriers [tear film (precorneal), corneal, conjunctival, blood aqueous, vitreoretinal interface to blood-retinal barrier] and the common route of administration. Primary methods of drug delivery to the eye are topical (1), local ocular [e.g., intravitreal (2) and subconjunctival (3)] and systemic [i.e., oral (4) and intravenous (5)]. Topical instillation is the most widely preferred non-invasive route of drug administration to treat diseases affecting the anterior segment and, potentially, the posterior segment. The ocular barriers block the entry of the most active molecules; hence, effective drug delivery systems are required to facilitate the passage of the drug across these barriers and transport the given pharmaceutical compound to its target site to achieve an optimal therapeutic effect [151] (Adapted from "Anatomy of the Human Eye", by BioRender.com (2022). Retrieved and edited from https://app.biorender.com/biorender-templates)

as elevated IOP, cataract formation, bleeding and the risk of ocular infections [159]. This route is particularly problematic because drugs can be prevented from reaching the target tissue by the posterior vitreoretinal interface, including the inner limiting membrane of the retina and the vitreous cortex [160]. Figure 3 shows a basic schematic of the eye with anatomical barriers and common routes of drug delivery.

The eyes are easily accessible in terms of delivering the drug into the body, yet the drug distribution is one of the most challenging endeavors. Therefore, the development of safer and more efficient drug delivery systems is vital for therapeutic purposes. Research conducted to date address these challenges and there is a growing consensus that the characteristics of ideal drug carriers are as follows [161, 162]:

- Particle size reduction and direct interaction with target cells or tissues (adhesive properties);
- Improved drug retention time in the precorneal area and promoted drug tissue permeation as well as optimal tissue absorption;
- Improved solubility of poorly soluble drugs (e.g., oral delivery of lipophilic drugs into the systemic circulation) and prolonged drug shelf-life;
- Biodegradability and biocompatibility;
- Absence of irritant features to reduce the drug dosing regimens and improve patient adherence to medication;
- Protection of sensitive therapeutic molecules (e.g., small molecule drugs and bioactive agents) against degradation agents such as enzymes;
- Targeted and controlled drug release characteristics that provide dose accuracy, reducing or preventing side effects and being ideal for long-term treatments.

The above criteria could be achieved by implementing NPs as drug carriers in ophthalmic drug formulations.

This platform transports the drug molecules across biological barriers by physically or chemically attaching the molecules to the NPs [163]. The large surface-to-volume ratio of NPs, along with other chemical characteristics, enables mucoadhesive properties that aid in drug adhesion to the mucosa of the corneal tissue, hence potentially increasing the drug contact time with the ocular tissue [164]. Apart from the abovementioned, NPs have been shown to improve patient self-care and compliance in terms of reducing the frequency of topical eye drop instillations, ultimately reducing the required doses and the risk of adverse effects [161]. NPs for ocular drug delivery can not only improve the solubility of drugs so as to reach the posterior segment of the eye, but also enhance the cellular uptake and protect the drug from degradation [165]. By formulating NPs with currently available ophthalmic solutions or investigational drugs, a greater potential for an effective glaucoma therapy can be ascertained in the future. Tabular overview of current investigated polymeric and lipid based-NPs with incorporated ophthalmic substances as compared to pure substances in ocular tissues is presented in Table 2.

Nanoparticles as ocular drug delivery systems

NPs are ultrafine solid structures that vary in morphology and have at least one spatial dimension in the range between 1 and 100 nm, and sometimes up to 500 nm, for larger particles. In the field of drug delivery, NPs are formulated to enhance the penetration and drug targeting of the active compound, while promoting a sustained release [166]. Due to their miniscule nature, NPs can often easily infiltrate the anatomical barriers in the CNS [164], such as the blood-brain and blood-retinal barriers, and thus directly provide a maximal drug bioavailability to the target cells [157]. In NPs, the drug-loading capacity is dependent on a few factors, including chemistry and microstructure, but also size, especially for NPs carrying their drug payload on the surface. Smaller NPs in these cases provide a higher loading capacity than the larger ones due to their higher specific surface area. NPs can exhibit a wide variety of morphologies, which help to serve the specific purposes to provide an effective therapy.

Multiple studies have attempted to develop drug-encapsulated NPs for the delivery to anterior and posterior segments of the eye. Conjugating ocular drugs onto NPs has been shown to boost eye permeation, particularly pass through the precorneal barrier [167]. In neurodegenerative diseases associated with inflammation, extensive studies have exploited drug-encapsulating NPs [168]. Some of the NPs that have been employed in neurodegenerative experimental studies, including polymeric and lipid based ones, have emerged as the key players in the domain of anti-inflammatory drug carriers [169].

Generally, in the drug delivery field for neurodegenerative and ocular diseases, NPs are most commonly made of soft carbonaceous materials, such as polymers and/or lipids [170]. Both lipid and polymeric NPs have successfully delivered drugs for several therapeutic purposes, while protecting the encapsulated drugs from enzymatic degradation and controlling their release. NPs made of natural or synthetic polymers and proteins [e.g., chitosan, poly(ethylene glycol) (PEG), polycaprolactone, sodium alginate, and albumin] usually take the form of finely dispersed latexes [171]. Compared to other nanomaterials such as the inorganic ones (e.g., zinc oxide or aluminum oxide), the former have caused a minimal eye irritation and prolonged retention of drugs, and thus allow for the circumvention of multiple medications and dose reduction [162]. However, compared to nanomicelles, a type of nanocarrier, polymeric-based NPs have been unable to escape the rapid loss of the instilled solution from the precorneal integument and the nasolacrimal drainage system. To overcome this limitation, NPs with mucoadhesive properties (i.e., chitosan and hyaluronic acid) were developed [172]. Of note, both polymeric- and lipid-based NPs have successfully delivered drugs for a number of therapeutic purposes, while protecting the encapsulated drugs from enzymatic degradation and controlling their release [166]. Figure 4 shows the benefits of drug loaded NPs administered through the corneal and blood-retinal barriers.

In the beginning of the application of NPs in ocular drug delivery, different carbon-based NPs were developed with the aim of producing a sustained drug release in the precorneal pocket. This was due to the majority of ophthalmic formulations being administered as eye drops, which in their conventional forms are poorly bioavailable on the corneal surface and intraocular tissues. Among the earliest NPs were those made of acrylic polymers such as poly-alkylcyanoacrylate (PACA), which extended the time of drug contact with the eye surface. There was increased drug action duration, however, this resulted in ocular toxicity. Later, polyacrylamide NPs began to replace PACA for the same purpose [173].

Polyester NPs (e.g., polycaprolactone) have emerged in the recent times as a key biodegradable material for ocular drug delivery, largely thanks to the acute tolerance of the ocular surface to them. At the same time, polyester NPs can increase drug efficacy. For example, an ophthalmic betaxolol, a beta-adrenergic blocking agent, displayed its optimal pharmacological effect when encapsulated by polycaprolactone (hydrophobic NPs) owing to its gradual release. NPs made of biodegradable materials such as hyaluronic acid or composed of hydrophilic

Table 2 Polymeric- and lipid-based conjugated NPs as carriers of ophthalmic substances

Nanoparticle's formulation	Substances	Size of NPs (nm)	Surface charge (mV)	Route of delivery	Platform	Advantages	Refs.
Polymeric-based NPs							
PLGA	Sparfloxacin	181 to 232	+22	Topical instillation (nanosuspension)	In vitro In vivo	Reduced IOP, improved precorneal residence time, enhanced ocular penetration, and good eye tolerance	[231]
PLGA coated-chitosan gel	Sparfloxacin	181	NR	Topical instillation (laden in situ gel)	In vivo	Reduced IOP, improved drug penetration, promoted sustained drug release, and prolonged drug retention time	[232]
PLA coated-PEG	Acyclovir	51.2 to 131.5	−14.7	Topical instillation (conjunctival sac)	In vitro In vivo	Reduced IOP, prolonged retention time, and improved drug efficacy	[233]
Poly(amidoamine) (PAMAM) coated-PLGA	Brimonidine tartrate; timolol maleate	258	−28.8	Topical instillation	In vitro In vivo	Reduced IOP (≥ 18%), non-toxic, prolonged time, increased drug bioavailability, controlled and slow release (~ 5 weeks)	[234]
PLGA-vitamin E-tocopheryl polyethylene glycol 1000 succinate	Brimonidine tartrate	115.72 ± 4.18	−11.80 ± 2.24	Topical instillation (in situ gel)	In vitro Ex vivo In vivo	Reduced IOP (~ 8 h), improved precorneal residence time, non-irritant, and sustained corneal release	[235]
PLGA-phosphatidylserine (PSA) (core shell NP)	Brinzolamide	571.00 ± 27.02	−27.45 ± 2.98	Subconjunctival injection	In vitro Ex vivo In vivo	Reduced IOP, enhanced corontial drug penetration, sustained release, high encapsulation efficiency, and non-toxic	[175]
Chitosan	Brimonidine tartrate	270 to 370	+26.2 to +29.8	Topical instillation	In vitro In vivo	Reduced IOP, non-irritant and safe, provided mucoadhesive effect, prolonged retention time, and sustained drug release	[236]
Chitosan coated-carbopol	Pilocarpine	294	+55.78	Topical instillation	In vitro In vivo	Prolonged drug release with high bioavailability (unloaded > 90% drug in ~ 4 h)	[237]
Chitosan coated-PLA	Rapamycin	300	+30.3	Topical instillation	In vitro In vivo	High precorneal retention time (50% within 12 h), prolonged drug release, and significant immunosuppressive effects	[238]

Table 2 (continued)

Nanoparticle's formulation	Substances	Size of NPs (nm)	Surface charge (mV)	Route of delivery	Platform	Advantages	Refs.
Chitosan coated-PLGA	Triamcinolone acetonide	334.00 ± 67.95 to 386.00 ± 15.14	+26 to +33	Topical instillation	In vitro	High drug encapsulation (55–57%) and controlled drug released (27 h)	[185]
Chitosan coated-sodium alginate	Gatifloxacin	205 to 572	+17.6 to +47.8	NR	In vitro	Rapid drug release in the first hour but prolonged release over 24 h	[239]
Chitosan coated-cyclodextrin	Econazole nitrate	90 to 673	+22 to +33	Conjunctival sac (instillation)	In vitro In vivo	Prolonged drug release (~50% within 8 h) and high bioavailability	[240]
Chitosan-coated sodium alginate/chitosan	5-Fluorouracil	329 to 505	+18.5 to +28.9	Topical instillation	In vitro In vivo	Increased drug bioavailability and prolonged release (~8 h)	[241]
Lecithin coated-chitosan	Natamycin	213	+43	Conjunctival sac (instillation)	In vitro In vivo	Increased drug retention time (> 64% released over ~7 h), reduced clearance, improved mucoadhesive properties, and fewer doses required	[242]
HA-modified chitosan	Timolol maleate; dorzolamide hydrochloride	118.4 to 143.9	+29.0 ± 8.7	Topical instillation	In vitro Ex vivo In vivo	Reduced IOP, improved mucoadhesive properties (~91%), provided controlled drug delivery, slow but sustained release, and non-irritant	[243]
Poly(γ-glutamic acid) (γ-PGA)-l-phenylalanine (-Phe)	Dexamethasone	200	− 25	Topical instillation	In vitro In vivo	Efficient drug uptake by cultured macrophages/microglia and inhibited microglia at 24 h post-treatment	[244]
Ethylcellulose	Melatonin	147.4 to 179.6	− 25 to − 30	Topical instillation	In vivo	Greater cornea penetration and RGC survival at 9 days post-treatment	[245]
Eudragit	Brimonidine tartrate	143.9 to 702.2	NR	Topical instillation	In vitro Ex vivo In vivo	Reduced IOP (~2 to 3 h longer than 1 h of commercialized eye drop) and prolonged drug release	[246]

Table 2 (continued)

Nanoparticle's formulation	Substances	Size of NPs (nm)	Surface charge (mV)	Route of delivery	Platform	Advantages	Refs.
Lipid-based NPs							
SLNs	Tobramycin	70 to 80	NR	Topical instillation (lower conjunctival sac)	In vivo	Prolonged drug release and retention (~4 h) and high bioavailability	[247]
	Baicalin	91.42 ± 1.02	−33.50 ± 1.28	Topical instillation	In vitro In vivo	Prolonged drug release (~62% after 3 h and the remaining gradually within 10 h) and high corneal permeability	[248]
SLNs-coated modified Chitosan	Methazolamide	143.9 to 702.2	+ 31.3 ± 1.7	Topical instillation	In vitro Ex vivo In vivo	High ocular penetration, sustained drug release (~8 h), fewer doses required, and enhanced patients' adherence	[249]
SLNs modified phospholipids	Timolol maleate	37.7 to 47.2	NR	NR	In vitro Human cornea construct	Enhanced drug bioavailability and encapsulation rate (> 44%)	[250]
SLN-PEGylated	Latanoprost	105 to 132	−29.1 to −26.7	Topical (contact lens)	In vitro In vivo	High drug uptake, sustained drug release, and safe	[251]
	Travoprost	221 to 257	−27.3 to −20.4	Topical (contact lens)	In vitro In vivo	High drug uptake, sustained release (> 144 h), safe and non-irritant	[178]
SLN-coated Poloxamer 188 and glycerol monostearate (solid lipid)	Chloramphenicol	248	−8.74	NR	In vitro	Increased encapsulation efficacy (> 83%) controlled and prolonged drug release (> 48 h)	[252]
SLN-coated glycerol monostearate	Bimatoprost	148.4 ± 1.25	−20.8 to −14.1	Topical instillation (in situ gel)	In vitro Ex vivo In vivo	Prolonged drug release, increase in precorneal residence time, non-irritant, safe with low corneal toxicity, and stable (> 1 month)	[253]
SLNs-coated Compritol 888	Indomethacin	140 ± 5	+ 21.0 ± 1.8	NR	In vitro	Increased drug stability, encapsulation (72%), and corneal permeability, stable (> 1 month)	[254]

Table 2 (continued)

Nanoparticle's formulation	Substances	Size of NPs (nm)	Surface charge (mV)	Route of delivery	Platform	Advantages	Refs.
NLCs	Mangiferin	51.39	−36.5 ± 1.5	Probe implantation	In vitro In vivo	Prolonged drug release (~ 3 months), increased corneal permeability and pericorneal retention time, high encapsulation efficacy (> 88%), and bioavailability	[255]
	Brimonidine	100 to 140	−31.1 to −33.7	Topical (contact lens)	In vitro In vivo	High drug uptake, sustained release (> 144 h), and safe	[177]
NLCs coated-Lutrol F 68 (surfactant), squalene (lipid) and Precirol ATO 5 (lipid)	Triamcinolone	198.73	−29.30 to −45.60	Conjunctival sac (instillation)	In vivo	No signs of ocular toxicity and improved encapsulation efficacy (94.82 ± 1.12%)	[256]
NLCs-coated Miglyol 812, castor oil, and stearic acid (lipid)	Flurbiprofen	228.3	−33.3	Topical instillation	In vitro In vivo	Prolonged drug release, high encapsulation efficacy (~ 90%) and minimal irritation	[257]
NLCs-coated Chitosan, with ethanol (co-surfactant), Tween 80 (surfactant), oleic acid (liquid lipid), and Compritol HD 5 ATO (solid lipid)	Ofloxacin	244	−4.630 ± 0.259	Topical (Ocular Inserts in *cul de sac*)	In vitro, microbiological test Ex vivo In vivo	Enhanced precorneal permeation, retention time (~ 24 h) and enhanced drug efficacy, and reduced frequency application	[258]
Lipid NPs coated-phospholipids	Diclofenac sodium	276	−12 to −42.6	NR	In vitro Human cornea construct	Increased drug encapsulation (~ 94%), corneal penetration, and prolonged drug release	[259]
Lipoamino acid-modified NPs	Connexin43 mimetic peptide	NR	NR	Intravitreal injection	In vivo	Enhanced neuroprotection after retinal ischemia	[260]

ATO = atomic grade; *IOP* = intraocular pressure; *NPs* = nanoparticles; *NR* = not reported; *PEG* = poly(ethylene glycol); *PLA* = poly(lactic) acid; *PLGA* = poly lactic-co-glycolic acid; *NLCs* = nanostructured lipid carriers; *SLN* = solid lipid NPs

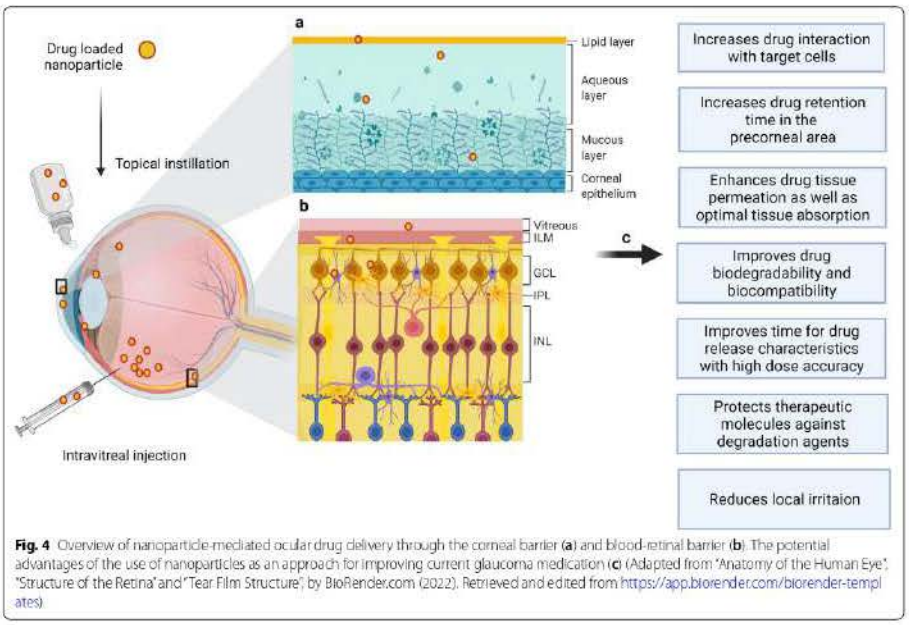

Fig. 4 Overview of nanoparticle-mediated ocular drug delivery through the corneal barrier (**a**) and blood-retinal barrier (**b**). The potential advantages of the use of nanoparticles as an approach for improving current glaucoma medication (**c**) (Adapted from "Anatomy of the Human Eye", "Structure of the Retina" and "Tear Film Structure", by BioRender.com (2022). Retrieved and edited from https://app.biorender.com/biorender-templates)

polysaccharides (components of the vitreous body) have also been proven safe for incorporation into ophthalmic solutions. Other NPs composed of polymeric-based materials have also improved the drug delivery interaction with the cornea, and thus allowed for the controlled drug release and the treatment of the ocular disease of the outer segment. It was postulated that nanocarriers coated with bioadhesive polymers (e.g., PACA and cyclosporine-A) can enhance the penetration of the embedded drug and improve the stability in the lacrimal fluid, which has been shown to prevent the enzymatic degradation of the delivered drug [173].

Recently, several studies have been performed to upgrade these standard NP formulations by reforming NP surface properties such as coating with functional groups [155]. One example is lectin, a glycoprotein that exhibits extremely high binding affinities for specific carbohydrate groups present on the surface of corneal epithelial cells [174]. Accordingly, better tissue penetration was demonstrated for positively charged NPs, unlike in the case of negatively charged NPs, which get electrostatically repelled from the cell membrane. To improve the adhesion on the mucosal surface e.g., at the periocular and oral mucosa, for a sustained drug release and efficient absorption, NPs were formulated with different bioadhesive polymers [173].

Due to their optimal size for the penetration of ocular barriers, NPs usually do not impose eye irritation, thereby limiting the frequency of drug administration as well as maintaining sustained drug release [161]. Lately, there has been an increased emergence of reports on NPs (e.g., polymeric [175, 176] and lipid-based [177, 178]) for drug-eluting contact lenses and corneal implants. Such commercialized medical devices provide a sustained and burst drug release with high bioavailability to the anterior and posterior segments of the eye, which may improve patient adherence as compared to eye drop medications [179]. Furthermore, contact lenses are typically used to correct refractive errors (e.g., myopia and hyperopia), which may positively impact patients' adherence towards their treatment regiment, particularly for those with both errors and glaucoma. Despite potential benefits, this approach is associated with potential safety risks and other limiting factors pertaining to production and storage [179]. As a result, topical eye drops continue to be the preferred first-line treatment option for glaucoma. Nonetheless, a substantial increase of studies is now being conducted to address the drawbacks of

drug-eluting contact lens, making it possible for delivering medications to the eye and commercialization in the future. Among many others, Chauhan et al. have been working extensively on developing novel loaded contact lenses employing diverse NPs formulations. While this is an intriguing topic, it is beyond the focus of this review. For further information on this subject, refer to the cited reviews by Chauhan and others [180–183] as well as their recent published works on the fabrication of ophthalmic drug-eluting contact lenses using various nanomaterials [180–182, 184–188].

In the posterior eye segment, prolonged drug delivery could be achieved with the application of NPs, depending on their size and characteristics of the surface. In addition, prolonged and effective transscleral drug delivery through intravenous administration (blood and lymph circulation) could be achieved by conjugating the right moieties onto NPs. Intravitreal administration, for example, enables macromolecular drugs to reach the retina and reduces systemic toxicities. Through this technique, the encapsulated drug molecules can be accumulated at the retinal pigment epithelium layer, and thus maximize the therapeutic effects [158]. Dexamethasone-loaded poly lactic-co-glycolic acid (PLGA) NPs administered intravitreally in rabbits, for example, elevated the cellular uptake with stable bioavailability in the vitreous fluid, chorioretina, and plasma compared to the unconjugated dexamethasone [189]. The same features were observed for human serum albumin NPs, where the conjugated drug molecules successfully infiltrated the retina layers through specific pathway in the Müller cells, relying on endocytosis and exocytosis [190]. Of note, more investigations are needed to ascertain the ocular tissue penetration of NPs loaded with high molecular weight drugs, especially through the vitreoretinal interface, which is one of the major obstacles to reach the inner retina upon intravitreal administration. Indeed, not all NPs allow the drug molecules to be efficiently dispersed through the vitreous fluid or pass through the inner limiting membrane. The efficiency of the therapeutics to cross this membrane are highly dependent on their ability to migrate from the injection site towards the retina, which is often determined by the size of the NPs along with their other physicochemical properties. For further reading on this subject, the reader is referred to the recent and ongoing work by Peynshaert et al. [50–52]. Nevertheless, in view of all that has been mentioned so far, one may suppose that NPs have the potential to serve as an effective intravitreal drug delivery system.

A few studies have been dedicated to the attempts to limit the drug clearance, given that most administered NPs pool in the liver and spleen and are removed by the reticuloendothelial system after the administration [191]. Interestingly, NPs are flexible and can escape opsonization by the macrophages if coated with extra surface layers, such as PEG [192]. By controlling properties of NPs, it is possible to achieve maximal therapeutic effects, minimal side effects as well as highest solubility for targeted drugs [192]. In order to prolong the drug retention and enhance its corneal permeability and bioavailability, it is important to select appropriate NPs in terms of their chemistry, size, shape, surface charge and other physicochemical properties [193]. Herein, the suitable biocompatible ocular drug delivery system depends on the target tissue, the route of administration, and the characteristics of the drug to be incorporated into the NPs. Despite all of the advantages, however, the high permeability of NPs pose a high risk, as shown in several brain studies [194]. For instance, zinc oxide NPs and the anatase phase of titanium dioxide can easily bypass the blood-brain barrier via multiple routes and induce neuroinflammation with the potential to be neurotoxic [195]. Hence, the careful selection of carriers is of prime importance when designing the drug delivery system utilizing NPs.

Application of different nanoparticles as anti-inflammatory drug carriers

Polymer-based NPs

Polymeric NPs have played a major role in the advancement of NP-mediated drug delivery, given that they have proven successful for alleviating numerous diseases [155]. Polymeric NPs can form nanocapsules (surface-vesicular systems) or nanospheres (matrix systems) depending on their internal structure and preparation method (Fig. 5). While the former systems contain a drug encapsulated within a liquid core cavity, the latter ones contain a structural polymeric matrix where the drug is physically and uniformly dispersed [196]. In addition to being incorporated inside a polymeric matrix, the drugs can also be adsorbed on the NP surface. These biodegradable NPs ranging from 10 to 100 nm are the most commonly studied in the ocular drug delivery field [173]. Polymeric NPs have been proven superior compared to other types of NPs for ocular application primarily due to their properties including biodegradability, lesser toxicity, similarity in stiffness compared to the soft tissues, good encapsulation capacity as well as controlled release manner, alongside biocompatibility and mucoadhesiveness [170]. Different types of polymeric NPs can be produced by directly processing different monomers or by using derived polymers obtained through polymerization [173]. They are also applicable in producing many different NPs, which can improve drawbacks of conventional drug delivery systems.

Polymeric NPs have been shown to improve the stability of easily volatile substances and may act as

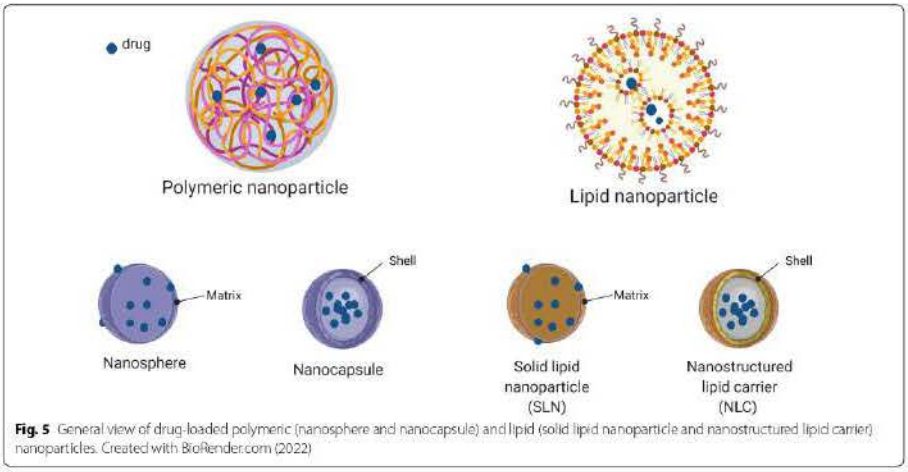

Fig. 5 General view of drug-loaded polymeric (nanosphere and nanocapsule) and lipid (solid lipid nanoparticle and nanostructured lipid carrier) nanoparticles. Created with BioRender.com (2022)

preservatives in ophthalmic solutions [197]. This results in a greater efficiency and effectiveness in transporting maximal concentrations of active pharmaceutical ingredients to the targeted site, making them an ideal choice for modifying drugs in cancer therapy. Apart from drugs, polymeric NPs have been used as gene delivery carriers [173]. Furthermore, high bioavailability of polymeric NPs is related to their ability to concentrate at a targeted spot via passive or ligand-mediated mechanisms. This method offers the possibility for reduction of required dose(s) and the side effects associated with them. Despite all these advantages, these types of NPs may still potentially cause toxicity due to the organic solvents incorporated in the final formulation and deterioration of the polymer, which may produce systemic pernicious aftermath effects [171]. Nevertheless, most polymeric NPs provide beneficial properties required for the use in drug delivery in ophthalmology, fundamentally relying on the capability to retain drugs in ocular tissues. By interacting with mucin, mucoadhesive polymers help to minimize the elimination of drugs from the surface of the eye and therefore can increase the drug bioavailability at the precorneal area. Several examples of these types of polymers with modified surface characteristics are PLGA, PEG, poloxamers, poloxamines, hyaluronic acid, chitosan, sodium alginate and polyacrylic acid [170].

One of the widely utilized materials for polymeric NPs is PLGA [198]. PLGA has been approved by the United States Food and Drug Administration (US FDA), owing to its high biodegradability and biocompatibility demonstrated by long-term clinical trials [199]. In in vitro studies, dexamethasone-encapsulated PLGA has been shown to successfully bypass the human placenta with high bioavailability [200]. An empirical study conducted using prednisolone on C6 cells, a type of cancer cell resembling astrocytes, has shown that prednisolone-encapsulated PLGA NPs attenuated pro-inflammatory cytokines including TNF-α and nitric oxide, surpassing the effects of naked prednisolone [201]. On the other hand, in a glaucoma study using the rabbit's cornea, a combination of dexamethasone and melatonin loaded PLGA NPs has been observed to significantly reduce the level of IOP [189]. This study associated the neuroprotective effect with the enhanced corneal penetration and sustained release of dexamethasone and melatonin by NPs. Interestingly, PLGA NPs are capable of encapsulating several active pharmacologic drugs simultaneously such as dexamethasone and melatonin for further improvement in delivering the drug [202]. For example, in an ex vivo rat brain tissue study, PLGA NPs coated with PEG exhibited rapid infiltration compared with uncoated NPs, indeed suggesting that coated NPs improved drug permeation [202].

In addition, prolonged delivery of therapeutic drugs is one of the most significant factors for successful neuroprotective therapy in glaucoma. To reach its target, NPs should be able to avoid the uptake by the mononuclear phagocytic system (MPS) of the host [203], which is responsible for opsonization and phagocytosis. Upon delivering the drugs, NPs are often specially designed

to avoid or harness the MPS to reduce inflammatory effects, subsequently improving the payload delivery and the drug therapeutic efficacy [203]. It is established that PEG-coated PLGA NPs improve drug uptake and clearance. For instance, hydrophobic PLGA NPs coated with hydrophilic PEG exhibited an antagonism against opsonization and phagocytosis along with prolonged circulation time in the blood compared with NPs prepared without PEG [204]. Moreover, in a study done using anthocyanin, a phenolic compound with a high antioxidant activity, PLGA-PEG NPs encapsulating this compound were shown to effectively abolish the expression levels of inflammatory markers including NF-kB, TNF-α, and inducible nitric oxide synthase (iNOS) as well as apoptotic markers such as bcl-2 associated x (Bax), b-cell lymphoma-2 (Bcl-2), and caspase-3 protein against amyloid beta peptide 1-42 (Aβ1-42)-induced neurodegenerative effects in SH-SY5Y cell lines [205]. Taken together, these PEG-coated PLGA NPs can improve therapeutic drug delivery by inhibiting both neuroinflammatory and neuroapoptotic pathways. Although the delivery of anti-inflammatory drugs with PLGA has yet to be studied in glaucoma, this highlights its strong potential as a nanocarrier, particularly for the treatment of neuroinflammation.

Lipid-based NPs

Lipid-based nanocarriers are at the forefront of the rapidly developing drug delivery systems for various diseases. Here, we hypothesize that the lipid-based NP system is also one of the most promising drug delivery systems for treating glaucoma, improving drawbacks associated with the conventional treatment. Topical liposomal nanocarrier is one widely used lipid-based nanocarrier in preclinical and early clinical studies, efficiently delivering ophthalmic solutions such as timolol maleate into the vitreous and retina [206]. In many respects, lipid NPs are superior carriers compared to liposomes and polymeric NPs. The main benefits of these NPs are that they do not require organic solvents, which generate toxic degradation products, to be formulated unlike the polymeric NPs. As a result, they exhibit low in vivo toxicity as well as protect and stabilize the loaded drug molecules from degradation, while offer the controlled drug release capacity.

Lipid NPs are composed of o/w (oil-in-water) emulsions, which is a combination of a lipid nucleus with an amphiphilic surfactant acting as the stabilizer. As such, they are able to transmit both hydrophilic or hydrophobic drugs [207]. Lipids in a liquid state can transform to a solid state of various structures (e.g., steroids, monoglycerides, diglycerides, and triglycerides) and be dispersed in an aqueous solution at room and body temperature. In this context, lipid NPs are the aqueous dispersion of spherical vesicles made of ionized lipids with a positive charge at the neutral pH. They range in size from 40 to 1000 nm and can be classified into two categories: solid lipid NPs (SLN) and nanostructured lipid carriers (NLC) (Fig. 5) [208].

Initially, SLNs were formulated to improve the available drug delivery systems such as polymeric NPs and liposomes. SLNs are made of solid lipids derived from water, co-emulsifiers, and emulsifiers. As the more improvised version of drug carriers than the aforementioned ones, SLNs deliver several advantages, including the improved drug loading capacity, prolonged duration of drug release, higher drug bioavailability with a better stability for unstable molecules against chemical degradation, improved safety as well as good cost-effectiveness ratio, particularly for high-scale production [207]. SLNs also enhance the corneal absorption and conjunctival uptake, as shown in studies done on anterior and posterior eye tissues, and thus extend the drug retention period [209–211]. These studies have shown the potential of implementing SLNs formulation in clinical practice.

Nanostructured lipid carriers (NLCs), on the other hand, were designed as the alternatives to compensate for the prominent drawbacks of SLNs, such as their very limited drug-loading capacity [212]. NLCs are formed from a mixture of solid and liquid lipids that adopts an amorphous solid matrix state at room and body temperature. NLCs exhibit high drug tolerance due to the physiological and biodegradable lipids constituting them. Moreover, NLCs offer a higher drug loading capacity and extended drug release time compared to the SLNs, which includes both hydrophilic and lipophilic drugs [207]. In general, there are three types of NLCs: the imperfect, non-shaped (amorphous) and the multiple structures. The imperfect type refers to a mixture of fatty acids blended to create several lipid formations in a crystal structure (disorganized matrix) with gaps which provide the space for lipophilic drugs to enter the particles. On the contrary, the amorphous type does not have a crystalline matrix, hence it prevents premature drug ejection. Lastly, the multiple structures type consist of several compartments of a liquid lipid in a matrix of a solid lipid. This NLC type is utilized to avoid drug decomposition caused by the solid lipid. The development of NLC formulations has been demonstrated in ocular drug delivery to both the posterior and anterior parts of the eye [213]. For example, Luo and co-authors reported NLC chitosan-coated genistein formulation delivered via a topical administration, which enhanced the transcorneal penetration with an increased bioavailability of the drug molecules in the aqueous humor compared to the conventional solution [214]. Furthermore, triamcinolone acetonide encapsulated

NLC showed enhanced therapeutic efficacy in mice. The developed formulation was able to reach the posterior segment of the eye via the corneal and non-corneal pathways upon topical administration [215]. These studies prove that the NLC formulations are good candidates for ocular drug delivery for treating glaucoma.

Overall, in comparison with other particulate systems, lipid NPs provide many advantages. From the commercial perspective, lipid NPs are feasible and easy to engage in a large-scale production [216]. Since lipids are biocompatible, lipid NPs are highly tolerated by the body. Furthermore, drug formulations with emulsifiers could have a better stability profile for both hydrophilic and lipophilic drugs, meaning they would be able to control and extend their retention time in the body. Besides, the important characteristics of lipid NPs including the optimal particle size, surface charge, drug entrapment efficiency, drug encapsulation and elimination, which enable them to protect the incorporated drugs from enzymatic degradation in the eye. This eventually provides a good adhesion onto the cornea/periocular tissues. Since they are composed of lipids, these NPs help to reach the lipid layer of the tear film, which directly improves the drug delivery and drug bioavailability for topical instillation. The natural affinity of lipid NPs for the lipid layers can be further augmented by endowing the given fatty acid chains with a positive surface charge, given that cationic lipids and surfactants extend the retention time of emulsion drops on the epithelial layer of the cornea [207]. Interestingly, studies done on the cytotoxicity of both SLNs and NLCs demonstrate that they are well tolerated and do not cause irritation to the ocular tissue. Still, non-ionic surfactants are occasionally required to minimize the toxic effects of the drug conjugated to lipid NPs [212]. Yet, these NPs can facilitate the passage of non-ionized drugs across various barriers, such as the precorneal film, while maintaining the neutral form of the encapsulated drug in its active form [212].

Conclusion

Several biological and nanoparticle-assisted agents have been evaluated in the experimental models of glaucoma, but none of them have passed the clinical trials. This possibly has to do with the complex molecular processes governing neuroprotection that are yet to be elucidated. Turbulence in the immune response surveillance is regarded as the prime source of the disease progression, including that in autoimmune and other neurodegenerative diseases. We, therefore, have discussed the prospect of tackling the inflammatory response at the early stages of glaucoma. Traditionally, the absence of the symptoms rarely prompts a clinical evaluation, let alone a treatment for the disease, notwithstanding the fact that most forms of glaucoma are asymptomatic in the early stages. It is also conceivable that IOP-lowering treatments may not be effective in circumventing the progression of the disease, as they lead to the progressive damage to the RGCs. The ineffectiveness of the available treatments has contributed to the improvement and development of several drug delivery systems. In ocular drug delivery systems, NPs have a vast applicability and potential to improve the efficacy of the current available treatments for glaucoma. NPs may enhance the current therapies by modulating drug solubility and subsequently enhancing bioavailability. They may also assist the drugs to permeate the critical barriers *en route* to their ocular target, but also extend the drug delivery timescale. The adverse effects at large may be minimized too as targeted delivery and improved bioavailability reduce the need for higher doses. Therefore, combining NPs with biological or small-molecule agents with the ability to counteract the inflammatory response in glaucomatous neurodegeneration can potentially move the field of glaucoma therapy forward.

Abbreviations

Aβ1-42: Amyloid beta peptide 1-42; AMD: Age-related macular degeneration; Ang II: Ang II angiotensin 1, 2; AP-1: Activator protein 1; AT1-R: Angiotensin type 1 receptor; Bax: Bcl-2 associated x; Bcl-2: B-cell lymphoma-2; BDNF: Brain-derived neurotrophic factor; C1, C3, C5, C1Q: Complement component 1, 3, 5, 1Q; CD3: Cluster of differentiation 3; CNS: Central nervous system; COX-2: Cyclooxygenase-2; EGCG: Epigallocatechin-gallate; ET-1: Endothelin-1; FasL: Fas ligand; GBE: Ginkgo biloba extract; GFAP: Glial fibrillary acidic protein; IL-1β, IL-6, IL-8, IL-18: Interleukin 1β, 6, 8, 18; iNOS: Inducible nitric oxide synthase; IOP: Intraocular pressure; IP10: Interferon gamma-induced protein 10; Jak: Janus kinase; MD: Mean deviation; MgAT: Magnesium acetyltaurate; MIP-1α, MIP-β; MIP-2: Macrophage inflammatory protein 1α, β, 2; MPS: Mononuclear phagocytic system; NF-κB: Nuclear factor kappa B; NLCs: Nanostructured lipid carriers; NLRP1, NLRP3: NOD-, LRR-family pyrin domain containing 1, 3; NMDA: N-Methyl-D-aspartate; NTG: Normal tension glaucoma; OHT: Ocular hypertension; ONH: Optic nerve head; PACA: Poly-alkylcyanoacrylate; PAMAM: Poly(amidoamine); PEG: Poly(ethylene glycol); PLGA: Poly lactic-co-glycolic acid; POAG: Primary open-angle glaucoma; NPs: Nanoparticles; RAAS: Renin-angiotensin aldosterone system; RGC: Retinal ganglion cell; ROS: Reactive oxygen species; RNFL: Retinal nerve fiber layer; RSV: Resveratrol; SIRT1: Sirtuin 1; SLNs: Solid lipid nanoparticles; SOD-2: Super oxide dismutase 2; TLR1, TLR4: Toll-like receptor 1, 4; TNF-α: Tumor necrosis factor-alpha.

Acknowledgements

Not applicable.

Author contributions

LL, NAAS, MZS, and AJAJ performed literature search and drafted the manuscript. SA, NAAN, VU, RK, and RM supervised and revised the manuscript. All authors contributed to the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grant from Ministry of Education, Malaysia (Grant Number: FRGS/1/2020/SKK06/USM/03/2).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Author WJ was employed by the company TardigradeNano LLC. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Received: 15 August 2021 **Accepted:** 9 June 2022
Published online: 02 July 2022

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Emerging Microbes & Infections

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/temi20

Comprehensive literature review of monkeypox

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To cite this article: Ma'mon M. Hatmal, Mohammad A. I. Al-Hatamleh, Amin N. Olaimat, Suhana Ahmad, Hanan Hasan, Nurfatihah Azlyna Ahmad Suhaimi, Khaled A. Albakri, Anas Abedalbaset Alzyoud, Ramlah Kadir & Rohimah Mohamud (2022) Comprehensive literature review of monkeypox, Emerging Microbes & Infections, 11:1, 2600-2631, DOI: 10.1080/22221751.2022.2132882

To link to this article: https://doi.org/10.1080/22221751.2022.2132882

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Emerging Microbes & Infections
2022, VOL. 11
https://doi.org/10.1080/22221751.2022.2132882

REVIEW

OPEN ACCESS

Comprehensive literature review of monkeypox

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ABSTRACT

The current outbreak of monkeypox (MPX) infection has emerged as a global matter of concern in the last few months. MPX is a zoonosis caused by the MPX virus (MPXV), which is one of the *Orthopoxvirus* species. Thus, it is similar to smallpox caused by the variola virus, and smallpox vaccines and drugs have been shown to be protective against MPX. Although MPX is not a new disease and is rarely fatal, the current multi-country MPX outbreak is unusual because it is occurring in countries that are not endemic for MPXV. In this work, we reviewed the extensive literature available on MPXV to summarize the available data on the major biological, clinical and epidemiological aspects of the virus and the important scientific findings. This review may be helpful in raising awareness of MPXV transmission, symptoms and signs, prevention and protective measures. It may also be of interest as a basis for performance of studies to further understand MPXV, with the goal of combating the current outbreak and boosting healthcare services and hygiene practices.

Trial registration: ClinicalTrials.gov identifier: NCT02977715..

Trial registration: ClinicalTrials.gov identifier: NCT03745131..

Trial registration: ClinicalTrials.gov identifier: NCT00728689..

Trial registration: ClinicalTrials.gov identifier: NCT02080767..

ARTICLE HISTORY Received 8 August 2022; Revised 6 September 2022; Accepted 2 October 2022

KEYWORDS Monkeypox; MPX; MPXV; viral outbreak; emerging infectious diseases

Introduction

Monkeypox virus (MPXV) is a member of a subset of the *Poxviridae* family called *Orthopoxvirus*. This virus causes infection with clinical presentation resembling smallpox (SPX), which is caused by infection with the variola virus (VARV). MPXV was first isolated in 1958 from laboratory monkeys with a pox-like disease in a Copenhagen research facility in Denmark [1,2]. Genomic studies have characterized MPXV into Central African/Congo Basin and West Africa clades with differential epidemiology and clinical manifestations [3]. Most MPXV outbreaks outside Africa come from the West Africa clades with less severe disease and primary infection [4].

Since MPX infection has a similar presentation to many pox-like diseases, diagnosis based on clinical observations alone is insufficient. Thus, real-time PCR is used to distinguish the two MPXV clades from other orthopoxviruses [5,6]. Coincident immunity against MPXV has been achieved through SPX vaccination due to shared genetic and antigenic properties. Since SPX eradication and hence vaccine cessation, waning herd immunity to orthopoxviruses has created an immunologically naïve population which, along with several other factors, has led to resurgence of MPXV [7].

Since the beginning of 2022, cases of MPX from several regions have been reported to the World Health Organization (WHO), indicating an alarming re-emergence of MPX. On 13 May 2022, the WHO confirmed a multi-country MPXV outbreak in Africa and non-endemic countries worldwide, especially in

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/22221751.2022.2132882.

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Europe. By 13 June 2022, the Pan American Health Organization (PAHO) and WHO (PAHO/WHO) had recorded a total of 1423 confirmed cases of MPX in 31 non-endemic countries with no deaths. Around 87% of these cases were reported in 23 countries in the European region [8]. Concern has grown about the ongoing MPX outbreak as there is a shortage of new reports, and this has led to proliferation of misleading information. The goal of this review is to examine the origin of MPX and its evolution, transmission, pathogenesis, diagnosis, epidemiology, host immunity, treatment and prevention.

Molecular basis of MPXV activity

The MPXV has a double-stranded DNA genome of 196,858 base pairs (bp) with around 200 genes [9]. As an orthopoxvirus, its genome contains two telomeres composed of identical but oppositely-oriented sequences of short tandem repeats [10]. This region of inverted terminal repeats (ITRs) makes up around 3% (6379 bp) of the MPXV genome and is involved in the replication and encapsidation of the genome [11,12]. Further details of the genomic organization of MPXV are shown in Figure 1.

MPXV encodes all transcription and replication enzymes needed for the viral genome [14]. It has been hypothesized that the progressive loss of genes not essential for human pathogenesis led to the emergence of a highly adapted virus that causes serious disease and is capable of efficient and rapid human-to-human transmission [15,16]. According to a study by Elde and colleagues, gene copy number variation may be a key element in regulating virus fitness [17]. Among the MPXV alignments, polymorphism in the noncoding region of the ITR with 12 variants was detected. Four of the 23 (17.4%) whole genome sequences displayed significant genomic instability just upstream of the right ITR. A 625-bp deletion between bases 189,820 and 190,444 was present in a collection of samples (genome locations based on MPXV-COG 2003 358). MPV-Z-N2R and the first 103 bp of the genus *Orthopoxvirus* major histocompatibility complex class I-like protein (OMCP) are both removed by this deletion. The function of MPV-Z-N2R is unknown, as neither the VARV nor the West African MPXV genomes contain any similar genes. OMCP is a secreted protein that binds to NKG2D and prevents natural killer cells from destroying infected cells [16].

Although MPXV is a DNA virus, its entire lifecycle occurs in the cytoplasm of infected cells, in which a variety of proteins needed for the replication machinery are encoded from open reading frames (ORFs) of the MPXV genome (Table 1). These ORFs have more than 90% sequence identity with those of other

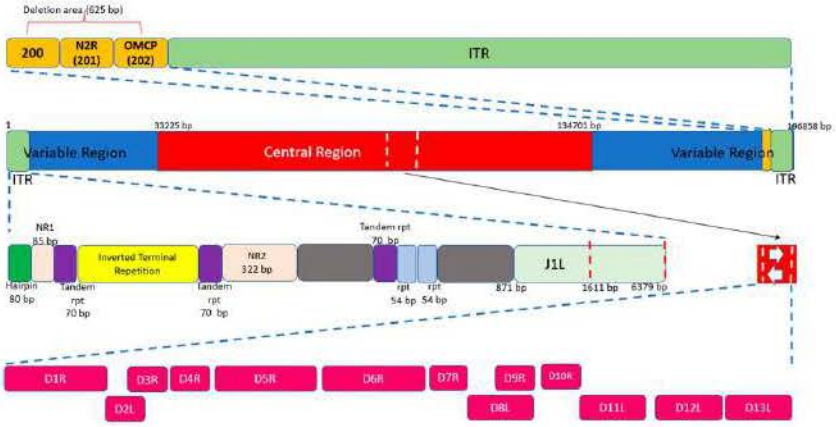

Figure 1. General structure of the MPXV genome. The genome is made up of double-stranded linear DNA (approximately 197 kb), primarily composed of hairpin loops, some open reading frames (ORFs), and tandem repeats, while the ITRs are made up of tandem repeats, hairpin loops, and some ORFs [13]. The ends of the genome form direct repeats called ITRs, and the genome has a terminal hairpin loop (no free ends). Most of the essential genes are located in the central part of the genome, and there are ~250 genes in the genome [14]. The upper box reveals a 625-bp deletion directly upstream of the right ITR (red box), which completely removes MPV-Z-N2R (locus 201) and truncates OMCP (MPV-Z-N3R, locus 202). The central part contains the following genes: D1R: large subunit of mRNA capping enzyme, D2R and D3R: internal structural proteins of intracellular mature virions (IMVs), D4R: viral DNA glycosylase, D5R: ATPase, D6R: subunit of early transcription protein, D7R: subunit of RNA polymerase, D8R: membrane protein of IMV, D9R, D10R, D11R: nucleotide triphosphate phosphorylate, D12R: small subunit of mRNA capping enzyme, and D13R: core protein of IMV [14].

Table 1. List of the most important ORFs in the MPXV genome and their functions.

ORF	Size	Functions
D5R	242	Zinc-binding, virulence factor and inhibition of UV-induced apoptosis
P1L	117	Secretion of virulence factor
C2L	375	Synthesis of serine protease inhibitor-like (SPI-3) and prevents cell fusion
C8L	151	Deoxyuridine triphosphatase production
C16L	439	Encoding serine/threonine protein kinase 2 and regulation of virion morphogenesis
C23R	101	Virion core DNA binding phosphoprotein
F1L	479	Poly(A) polymerase and catalytic subunit
F3L	153	dsRNA binding inhibits dsRNA-dependent protein kinase and 2-5A-synthetase
F4L	259	RNA polymerase, 30-kDa subunit and intermediate stage transcription factor
F8L	1006	DNA polymerase
Q2L	108	Virion-associated glutaredoxin
I1L	312	Virosomal protein needed for virus multiplication
I3L	269	ssDNA-binding P-protein interacts with R2 subunit of ribonucleotide reductase
I4L	771	ssDNA-binding P-protein interacts with R1 subunit of ribonucleotide reductase
I7L	423	Virion core protein, DNA topoisomerase II homologue from
G4L	124	Virion-associated glutaredoxin, required for disulphide bonds and assembly
H5R	213	Virosome-associated, late gene transcription factor, VLTF-4, $Ca2^+$-binding motif
E5R	785	Nucleic acid-independent nucleoside triphosphatase, required for DNA replication
A11L	891	Major virion core protein p4a
A13L	190	Virion core protein
A19R	492	DNA helicase, post replicative negative transcription elongation factor
A34L	300	DNA packaging into virion and NTP-binding motif A
A50R	554	DNA ligase

Source: Adapted from Shchelkunov et al. [9].

orthopoxviruses. The majority of species- and strain-specific differences between orthopoxviruses are in the left and right terminal regions [9].

Some viral proteins have been found to be essential components of MPX. These proteins are classified into three categories: (1) viral entry proteins that facilitate MPXV entry into host cells through receptor binding and membrane fusion; (2) viral proteins that facilitate release of MPXV copies from host cells; and (3) essential proteins for modulation of the host cell and immune modulation. These proteins are summarized in Table 2 and their roles in host cells are further discussed in the next sections.

Like other orthopoxviruses, the pattern of MPXV gene distribution has the genes that encode for housekeeping functions conserved and clustered in the central region of the genome (Figure 1), whereas those that encode for proteins involved in virus-host interactions are less conserved and located in the terminal regions [13,18–23]. It has been hypothesized that MPXV is a direct ancestor of VARV based on the similarity in the clinical manifestation of the two diseases [24–26]. Later studies confirmed this hypothesis by detecting high similarity in genetic material between the two viruses using genomic restriction endonuclease maps [27,28] and nucleotide sequencing [29,30]. However, other studies have postulated the independent evolution of the viruses [27,31]. Whole genome sequencing has shown that MPXV is not a direct ancestor nor a direct descendant of VARV [32].

The genetic diversity between the West African and Congo Basin clades has been documented in several studies of the evolutionary relationships between the clades [33]. The clades are 99.4% identical at the protein level, but include several functionally unique genes, non-functional ORF regions and additional ORFs [33].

During the multi-country 2022 outbreak, several preliminary phylogenetic analyses of MPXV genomes were performed from samples collected in Portugal, Belgium, France, Germany, the Netherlands, Italy, Spain, Slovenia and Brazil. The data confirmed that the West African MPXV genotype is central to the ongoing outbreak. A total of 117 MPXV sequences were identified up to 24 June 2022 (Supplemental Table 1) using the NCBI database related to the current outbreak of MPXV outside the endemic area. Notably, detection of MPXV was correlated with individuals who had returned from the Canary Islands [34], Slovenia [35], Italy [36] and France [37].

The sequences from Slovenia were from two patients who presented with anogenital skin lesions, swollen inguinal nodes and malaise. Skin sampling was used to isolate the MPXV genome from the French sample. Nasopharyngeal swabs, lesion crust and vesicles were used as viral genetic material harvested after one week-onset of mild symptoms, including fever and odynophagia, from the sample from the Canaries. Sequences were also obtained from a 30-year-old male who presented in Belgium with perianal papules and a 1-cm painful inguinal adenopathy bilaterally after travelling to Lisbon, Portugal [38]. A 41-year-old male patient was diagnosed with MPX after a trip to Portugal, Spain and Brazil [39].

The greatest number of sequences (50) was from a study in Germany, in which whole genome sequencing was used for samples from a 26-year-old patient who presented with acute symptoms of orthopoxviral infection [40]. A further 28 sequences were detected using the paired-end sequencing technique in Portugal [41].

Most MPXV sequence isolates have been obtained from male patients from Belgium [38], Portugal [42], Italy [36,43], Brazil [39] and Spain [44]. Infection with human immunodeficiency virus (HIV) was reported in two cases: a 31-year-old Spanish man [44] and a 39-year-old Italian man who had HIV infection with a history of unprotected sex with male partners [36].

A number of novel single nucleotide polymorphisms (SNPs) among newly-detected MPXV sequences have been identified during the 2022 outbreak, including 46 SNPs in newly-discovered MPXV sequences from Spain, in comparison with genomes from the

Table 2. List of known MPXV proteins, their encoding genes and host target proteins.

Gene	Protein	Host target proteins
Entry proteins		
M1R	Protein L1 (virion membrane protein)	Probably binds to host cell entry receptors
E8L	E8L (cell surface-binding protein)	Cell surface chondroitin sulphate proteoglycans (CSPG)
H3L	H3L (envelope protein)	Cell surface heparan sulphate (HS)
Exit proteins		
A38R	IEV (transmembrane phosphoprotein)	NCK adaptor protein 1 (NCK1), kinesin light chain 1 (KLC1), intersectin-1 (ITSN1) and epidermal growth factor receptor substrate 15 (EPS15)
C23R	Core phosphoprotein F17	Rapamycin-insensitive companion of mTOR (RICTOR) and regulatory-associated protein of mTOR (RPTOR)
C18L	Protein F12	Kinesin light chain 2 (KLC2)
Immunomodulatory proteins		
J3R	Chemokine binding protein	CC and CXC chemokines
J2L	Cytokine response-modifying protein B	Tumour necrosis factor-alpha (TNF-α), TNF-β, CC motif chemokine ligand 28 (CCL28), CCL25, CXC motif chemokine ligand 12 (CXCL12), CXCL13 and CXCL14
D9L	Ankyrin repeat domain containing protein CP77 (type I interferon (IFN) evasion protein)	Cullin-1 (CUL1) in the SKP1-CUL1-F-Box (SCF) complex
F3L	RNA-binding protein E3	Interferon-stimulated gene 15 (ISG15), eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2)/protein kinase R (PKR) and Z-DNA binding protein 1 (ZBP1)
H1L	Dual specificity protein phosphatase H1	Signal transducer and activator of transcription 1 (STAT1)
D3R	EGFR binding protein (MPXgp006)	Epidermal growth factor receptor (EGFR)
D11L	Protein C6	TRAF family member associated NF-κB activator (TANK), TANK-binding kinase 1-binding protein 1 (TBKBP1), 5-azacytidine-induced protein 2 (AZI2) and STAT2
C7L	Protein F1	Bcl-2-like protein 11 (BCL2L11), NLR family pyrin domain containing 1 (NLRP1) and Bcl-2 homologous antagonist/killer (BAK)
B16R	Soluble IFN-alpha receptor	IFN-α
C1L	IFN antagonist K1L	IFN
B13R	Protein B13	IκB kinase β (IKKβ)
B9R	Soluble IFN-γ receptor B8	IFN-γ
P1L	Protein N1	Bcl-2-associated agonist of cell death (BAD) and Bcl-2-associated X protein (BAX)
C6R	Protein K7	DEAD-box helicase 3 X-linked (DDX3X)
A37R	MHC modulating protein	Major histocompatibility complex (MHC) class II
A41L	Protein A41 (chemokine binding protein)	CCL21, CCL25, CCL26 and CCL28
A47R	TLR inactivating protein (MPXgp157)	Myeloid differentiation factor-88 (MyD88) adaptor-like and TIR-domain containing adapter-inducing interferon-β (TRIF)-related adaptor molecule (TRAM)

Data were collected from https://viralzone.expasy.org/9976 and https://www.uniprot.org/.

2018/2019 outbreak [34]. Six SNPs were identified between the two draft genomes from Madrid [44] and 6 from Italian sequences, in comparison to other MPXV genomes detected during the 2022 outbreak.

Microevolution of MPXV may explain the newly-detected clusters of viral genomes during the 2022 outbreak caused by the emergence of 7 SNPs leading to further subclusters and sub-branching from the common ancestor [41]. A frameshift deletion of 913 bp in the viral genome has been reported in two sequences from Portugal [41]. The effect of the number of SNPs detected in this genome compared to those isolated in the UK during the 2018–2019 outbreak led to synonymous, missense, stop-gained and intergenic variants [41]. These microevolution events enhance the evidence of human-to-human transmission of MPXV strains evolved from the West African ancestor of the MPXV currently detected outside the endemic area [16]. The MPXV isolated from the 2022 outbreak seems to have more mutations, but many of these newly acquired mutations have unclear function and significance [45]. These mutations could be the underlying cause of the sudden emergence of MPX cases in non-endemic areas. However, this can be ruled out because DNA viruses have lower per-site mutation rates due to the extensive interactions between viral DNA genomes and cellular pathways that detect and repair DNA damage, compared to RNA viruses (e.g. HIV and SARS-CoV-2) [46]. Therefore, further studies are required to determine the mechanism of action of these mutations.

Pathogenesis

The pathogenesis and mechanism of action of MPXV are similar to those of VARV and *Vaccinia* virus (VACV) [47]. MPXV, like other poxviruses, probably infects a wide range of mammalian cells without the need for specific host receptors and molecules for cell entry and replication [48]. The infection process begins with binding and entry of the extracellular enveloped virus (EEV) virions into the host cell through interactions of MPXV surface proteins with primary attachment receptors (glycosaminoglycans) on the cellular membrane of host cells [49]. Information on specific MPXV proteins involved in host cell entry and the receptors on host cells is currently lacking, but three proteins have been identified as viral entry proteins that may facilitate MPXV entry into host cells through receptor binding and membrane fusion. The first is protein L1, a virus membrane protein that probably binds to host cell entry receptors. The specific roles of protein L1 during MPXV entry are unconfirmed, but studies on VACV show that this envelope protein binds to the cell surface

through the entry/fusion complex (EFC) and is required for merging the virus membrane to the host cell membrane during viral penetration [50–52]. E8L is another MPXV cell surface-binding protein that is suggested to bind to host cell surface chondroitin sulphate proteoglycans (CSPG) and mediate adsorption of intracellular mature virus (IMV) virions to cells [53]. The MPXV envelope protein H3L has also been studied in *in-vitro* and *in-vivo* on VARV, indicating important roles for this protein in virus adsorption to cell surface heparan sulphate and IMV morphogenesis [54]. Despite the variability in surface glycoproteins and the number of wrapping membranes between the IMV and EEV virions [55], IMVs that exit infected cells through budding can also penetrate the cellular membrane and infect other host cells, but less efficiently than EEV [56].

After fusion of MPXV EEV or IMV with the cellular membrane, the internal virion components are spontaneously uncoated with loss of viral membranes and enter the host cytoplasm [57]. All poxviruses replicate their nucleic acid exclusively in the cytoplasm and encode proteins that facilitate genome replication and gene expression [58]. The cytoplasmic replication cycle of MPXV is a complex sequence of events that needs further investigation, but the intracellular cycle of MPXV can be visualized based on understanding of VACV replication as the best-studied poxvirus (Figure 2).

The first attempt at understanding the pathogenesis of MPXV was made in 1969 by Wenner et al. [63]. Cynomolgus monkeys were infected with MPXV intramuscularly and the virus started to multiply in local cellular components at the injection site. In addition to detection of MPXV at the site of inoculation, an intense inflammatory immunoresponse is seen in cell necrosis, phagocytosis, vasculitis and local replication of MPXV [63]. Primary viremia then developed based on detection of the virus in regional lymphatic and vascular channels. MPXV is transported in lymph to regional lymph nodes and very likely in blood to the spleen, tonsils and bone marrow. These organs, among others, comprise secondary sites of virus multiplication and with further release of the virus, there is a consistently measurable level of viremia. At this stage, it is likely that the virus is transported to tertiary target organs, including the skin and testes, resulting in clinically recognizable disease.

The difficulty in understanding the pathogenesis of MPXV is due to the lack of ideal animal models with routes of MPXV transmission similar to those in humans, similar pathways of pathogenesis, and similar rates of infection, morbidity and mortality [64,65]. The main challenge is the resistance and non-infectivity of the virus in commonly used animal models, such as guinea pigs and golden hamsters [64,66]. However, *in-vitro* investigations of the kinetics of the poxvirus replication cycle using different cell lines have indicated similarity of MPXV with VACV and VARV in the production of viral antigens, patterns of cytopathological change and formation of inclusion bodies, and release of new virions from host cells [67–71]. Despite some differences among poxviruses based on the type of cell culture lineage and cell growth conditions, a better understanding of MPXV has emerged based on VACV studies.

The first step in MPXV replication following inoculation is virion attachment to the target cells, which has been investigated *in vitro* using rhesus monkey kidney cells or kappa cell lines, in which up to 85% of virion particles are found to attach within 2 h [72]. Synthesis of messenger RNA has a vital role in the uncoating process [47].

Using 2 plaque-forming units (PFU)/cell to infect CV-1 cells reveals a 6-h period of partial eclipse, presumably representing the period of attachment, uncoating and synthesis of the earliest virions. The pattern of increase of cell-free virus follows closely with that of cell-associated virus, with a lag of 3 or 4 h between intracellular maturation and extracellular release [72,73]. The newly synthesized virus releases from kappa cells at a rate of 1% [72] and from CV-1 cells at 10% [73]. MPXV antigens can be detected in nuclear region or long cellular bridges of infected cells using cytoplasmic immunofluorescence [73].

Cytopathic effects of MPXV have been observed in primary and secondary lines of kidney cells derived from rhesus, cynomolgus and African green monkeys [72–75], bovine, rabbit and guinea pig kidneys, mouse liver cells [33,72] and human-derived amnionic and lung fibroblasts [75,76]. Cytopathic effects have not been reported for all cell lines, but a few lines in HeLa cells, chicken embryo and other cell cultures have shown these effects [72,76,77]. Granulation, rounding up and cellular condensation have been reported as cytopathic effects caused by MPXV, and monolayer cells detached from the side of the glass, leaving microscopic visible "holes." Affected cells in monkey kidney and human amnion cell cultures are interconnected by thread-like syncytial elongations, but such cellular bridges are not apparent in HeLa cells [75].

Depending on the size of the inoculum, CPE- of MPXV-infected CV-1 cells (a continuous line of African green monkey kidney cells) may be observed as early as 8 h or as late as 10 days or more [73]. When a suspension of pustular material from infected monkeys is inoculated into such tissue cultures, the CPE usually develops in 2–3 days. Complete destruction occurs after 5 days of incubation [75]. In tissue cultures, the infectivity titres of most passage fluids vary between 10^{-4} and 10^{-6} for the 50% tissue culture infective dose ($TCID_{50}$) [73,75]. The physical

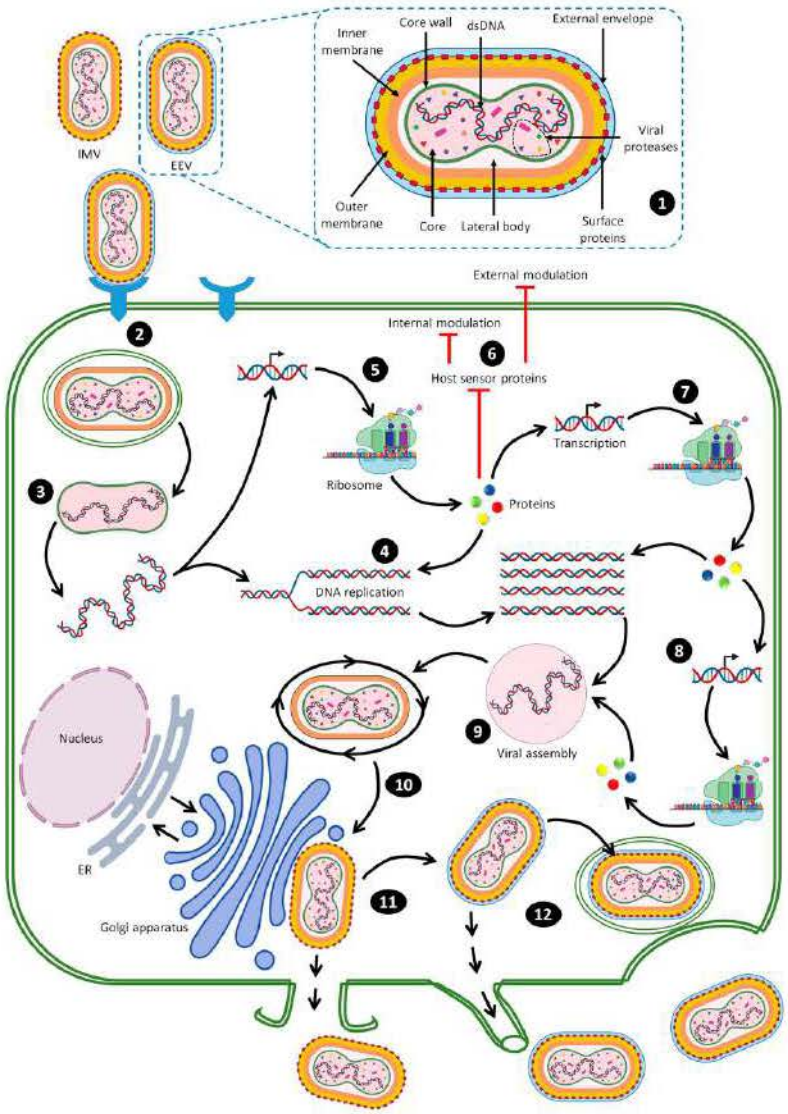

Figure 2. Steps of MPXV entry into host cells [13,14,59–62]. (1) Schematic of the structure of MPXV. (2) Both the EEV and IMV virions penetrate the host membrane by binding and macropinocytosis. MPXV virions use glycosaminoglycans as host receptors. (3) After the internal virion components enter the cytoplasm, core uncoating occurs and this process leads to delivery of the MPXV genome and accessory proteins to the cytosol. (4) The released MPXV genome is used as a template for DNA replication. (5) Early viral DNA transcription followed by translation into the host ribosome occurs to encode essential proteins. Early proteins aid in DNA replication. (6) These proteins interact with host sensor proteins resulting in internal and external modulations. The major intracellular modulations include prevention of viral genome detection, induction of cell cycle arrest, apoptosis inhibition, inhibition of the antiviral system and modulation of some host cellular signalling pathways. Early proteins play essential extracellular roles as immunomodulatory agents and as growth factor-like domains that stimulate onset of mitosis in neighbouring cells. (7) Early proteins are used in production of intermediate proteins. (8) These proteins are involved in late transcription and translation processes and aid in DNA replication. (9) Late proteins are essential components for viral assembly. (10) Viral morphogenesis occurs by formation of inner tubular nucleocapsid structure folding and assembly of viral glycoproteins to generate MV virions. (11) Except those released via infected cell lysis, MV virions transit to the Golgi apparatus along microtubules for double membrane wrapping. (12) The resulting EEV virions exit the infected cell by two routes: by the actin tail assembly, which provides enough force to propel the virions out of the cell or by budding from a cellular membrane (Created with BioRender.com).

characteristics of CPE produced by MPXV in monkey kidney-cultured cells cannot be distinguished from those of VARV [77] or VACV [73].

In addition to cytopathic effects, plaque formation with MPXV has been detected in various cell culture lines [72,73,78]. The plaque formation assay is a quantitative method, in which monolayers of monkey kidney cells infected with MPXV are allowed to overgrow and then stained using neutral red to demonstrate well-defined plaques of 2–3 mm in diameter [72,73,79]. Previously, MPXV was differentiated from VARV by the smaller size of the plaques [78] and by the ability of MPXV to form plaques in chicken embryo fibroblasts [78,80].

As viruses are intracellular and host-dependent microorganisms [81], survival inside infected host cells is critical for virus propagation, and this is based on manipulation of host cell signalling pathways. This manipulation enhances the viral replication cycle and determines disease outcomes [82], mainly by targeting cell growth and immunoregulation [83]. Thus, orthopoxviruses can inhibit cell apoptosis and the antiviral host defence, and exploit the host cell machinery [84].

MPXV immunomodulatory proteins and related immune responses

As shown in Table 2, a variety of MPXV proteins are implicated in host immunomodulation after being encoded in host cells. The chemokine binding protein encoded by the MPXV *J3R* gene binds to CC and CXC chemokines with high affinity, regulating leukocyte trafficking to tissues infected with MPXV and thus reducing viral virulence and inflammatory response [85,86]. Protein A41 is another chemokine binding protein that is encoded by *A41L* and targets the CC motif chemokine ligands CCL21, CCL25, CCL26 and CCL28. Bahar et al. [87] suggested that A41 forms sufficient interactions with these chemokines to prevent chemokine-glycosaminoglycan interactions at the cell surface, thereby destroying the chemokine concentration gradient and ultimately resulting in decreased neutrophil migration in tissues infected with MPXV [87,88]. The MPXV-encoded cytokine response-modifying protein B (CrmB) helps the virus to evade host immune defence by binding to host tumour necrosis factor (TNF) as a soluble decoy TNF receptor (TNFR) [89]. Thanks to its C-terminal domain, CrmB also binds to CCL28, CCL25, CXC motif chemokine ligand 12 (CXCL12), CXCL13 and CXCL14, with binding affinities comparable to those of TNF [89].

MPXV also encodes Ankyrin repeat domain containing protein CP77, which plays an early role in evading the antiviral state induced by type I interferon (IFN) by binding to cullin-1 (CUL1) in the SKP1-CUL1-F-Box (SCF) complex [90]. The SCF is an ubiquitin-protein ligase complex. It has been suggested that, following C-terminal phosphorylation, IFN regulatory factor 3 (IRF3) is recognized by CUL1, which is part of the SCF complex [91]. This leads to its polyubiquitination and targeting of the proteasome, indicating a significant role for the SCF complex in controlling IRF-3 stability [91]. IRF3 controls multiple IFN-inducing intracellular pathways that are triggered by RNA and DNA sensors [92]. Two MPXV genes, *B16R* and *B9R*, encode proteins that mimic the soluble IFN-α and IFN-γ receptors, respectively. These two proteins bind to IFN-α and IFN-γ to block the functions of IFNs, thereby inhibiting defences against MPX infection [93,94]. Also, the MPXV K7 protein binds to DEAD-box helicase 3 (DDX3) and inhibits *IFN-β* promoter induction [95]. DDX3 is a multifunctional protein involved in RNA metabolism and plays an essential role in key cellular biogenesis processes [96]. A recent study showed that DDX3 has a critical role in promoting *IFN-β* transcription formed by antiviral signalling by enhancing IRF-3/p300 holocomplex binding to the *IFN-β* promoter [97].

IFN antagonist K1L is another protein encoded by MPXV that inhibits the IFN-induced antiviral system [98]. K1L may not block IFN signalling pathways directly, but it prevents acetylation of the p65/RelA subunit of nuclear factor kappa B (NF-κB) [99]. NF-κB signalling is involved in regulation of major immune functions, especially by inducing antiviral genes such as IFN and IFN-stimulated genes (ISGs) [100]. MPXV also encodes the B13 protein that binds IκB kinase β (IKKβ), which contributes to IκBα phosphorylation and NF-κB activation [101], ultimately resulting in blocking the NF-κB signalling pathway by inhibiting IKKβ dimer trans-autophosphorylation as part of the activation mechanism [102].

MPXV encodes the dual specificity protein phosphatase H1 (H1L), which is involved in viral replication [103] and also has a role in immune evasion by blocking IFN-induced antiviral immune responses by dephosphorylating signal transducer and activator of transcription 1 (STAT1). It has been suggested that H1L can also block expression of STAT1-dependent and STAT1-independent genes [104]. STAT1 has an essential role in controlling expression of human IFNs and thus the severity of viral infections [105]. STAT1 is also involved in immunoglobulin (Ig) class switch recombination (CSR) and in production of memory B cells that contribute to tissue-resident humoral immunological memory by controlling the IgG response against viral reinfection [106].

MPXV protein C6 works as a viral immunomodulator by binding to the transactivation domain STAT2. This association decreases STAT2 phosphorylation and results in blocking of IFN signalling pathways

[107] and forms an integral part of the transcriptional responses to IFNs [108]. MPXV protein C6 also binds TRAF family member associated NF-κB activator (TANK) and inhibits IFN regulatory factors 3 and 7 (IRF3 and IRF7) [109]. IFRs are transcription factors that play crucial roles in several innate and adaptive immune responses, including the antiviral state and regulation of immune cell differentiation. These proteins are key regulators of induction of IFN gene expression downstream of pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), which recognize viral nucleic acid [92]. The nature of the signalling complexes formed on regulation by IRFs leads to further targets for MPXV protein C6. Thus, C6 binds to TANK-binding kinase 1-binding protein 1 (TBKBP1), which is an adaptor protein that binds to TBK1 and inhibits activation of IRF3 and IRF7 [109]. Since TBKBP1 is part of the interaction network in the TNF and NF-κB pathway [110], protein C6 may contribute to MPXV immune evasion via other cellular pathways.

MPXV A47R protein also inhibits the TLR signalling pathway by targeting myeloid differentiation factor-88 (MyD88) and TIR-domain containing adapter-inducing interferon-β (TRIF)-related adaptor molecule (TRAM), which are well-known as adaptors for inflammatory signalling pathways downstream of members of the TLR family [111]. MPXV evades host immune defence using the A37R protein, which targets MHC class II and suppresses the MHC class II antigen presentation pathway by affecting the stability or intracellular sorting of these proteins [112]. Class II MHC proteins facilitate the presentation of viral proteins found in the cytoplasm and exocytic compartments after macroautophagy by antigen-presenting cells (APCs) [113].

To counteract the viral inflammatory response, MPXV encodes RNA-binding protein E3, which inhibits ISG15 [114] and targets eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2)/protein kinase R (PKR) [114] as a crucial enzyme for regulation of the integrity of newly synthesized IFN mRNA [115]. E3 also targets Z-DNA binding protein 1 (ZBP1), also known as DNA-dependent activator of IFN regulatory factors (DAI) [116], which works as a cytoplasmic DNA sensor and functions in the development of immune responses [117]. ZBP1 plays a crucial role in controlling virus replication, and deletion of ZBP1 is significantly associated with severe viral infections [117,118]. ZBP1 binds to the receptor-interacting protein kinase 3 (RIP3) to form a complex that mediates virus-induced programmed necrosis [119].

MPXV uses protein F1 in targeting the nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) receptor (NLR) proteins and binds NLR family pyrin domain containing 1 (NLRP1) [120]. NLRP1 is involved in the formation of inflammasomes as important cytosolic multiprotein oligomers of the innate immune system [121]. Caspase-1 cysteine protease activation by inflammasomes is a crucial immune response to viral infections, as it stimulates production of IL-1β, IL-18 and high mobility group box 1 (HMGB1) protein to initiate pronounced inflammatory responses. Caspase-1 also triggers pyroptosis of host cells to eliminate the virus [122]. It may thus be beneficial to MPXV to hijack the inflammasome machinery and inhibit caspase-1 activation by targeting NLRP1 with protein F1.

MPXV proteins F1 and N1 target the B-cell lymphoma 2 (Bcl-2) family of proteins that control cellular apoptosis. Through the proapoptotic Bcl-2 homology 3 (BH3) domain, F1 binds host pro-apoptotic Bcl-2-like protein 11 (BCL2L11) and Bcl-2 homologous antagonist/killer (BAK) [123], while N1 binds Bcl-2-associated agonist of cell death (BAD) and Bcl-2-associated X protein (BAX) [124,125]. Since BCL2L11, BAK, BAD and BAX are all involved in altering apoptosis and autophagy by elimination of BH3-only proteins [126–128], both F1 and N1 may have anti-apoptotic roles in MPX infection. Bcl-2 has also emerged as a regulator of innate immune responses [129].

MPXV encodes protein MPXgp006 containing an epidermal growth factor (EGF)-like domain that targets the EGF receptor (EGFR). This domain binds an ErbB protein containing four receptor tyrosine kinases and is structurally related to EGFR [130]. The EGFR signalling pathway is among the most crucial in mammalian cells and involves complex processes that regulate a wide range of essential cellular functions such as apoptosis, differentiation and proliferation. This pathway also has a role in regulating intercellular communication [131]. Thus, MPXV relies on EGFR-regulated pathways to invade host cells and turn them into virus-making factories [132]. A previous study on VACV confirmed that poxviruses hijack EGFR-induced cell motility to enhance efficient virus spread and pathogenesis [133].

MPXV immunomodulatory proteins can be subdivided by function into three distinct categories: virostealth, virotransduction and viromimicry (Figure 3). The virostealth proteins act intracellularly, reducing detection of signals of MPX infection by interfering with host signalling processes, which results in a decrease in the capacity of cell-mediated immune responses (cytotoxic T cells) to recognize and destroy virus-infected cells. The virotransducer proteins also act intracellularly to inhibit innate antiviral signalling pathways and apoptotic responses to MPX infection. Viromimetics (virokines and viroceptors) are the only type of MPXV proteins that have extracellular roles [134,135]. Both types of viromimicry proteins are involved in regulating antiviral immune responses. Viroreceptors are expressed as cell surface

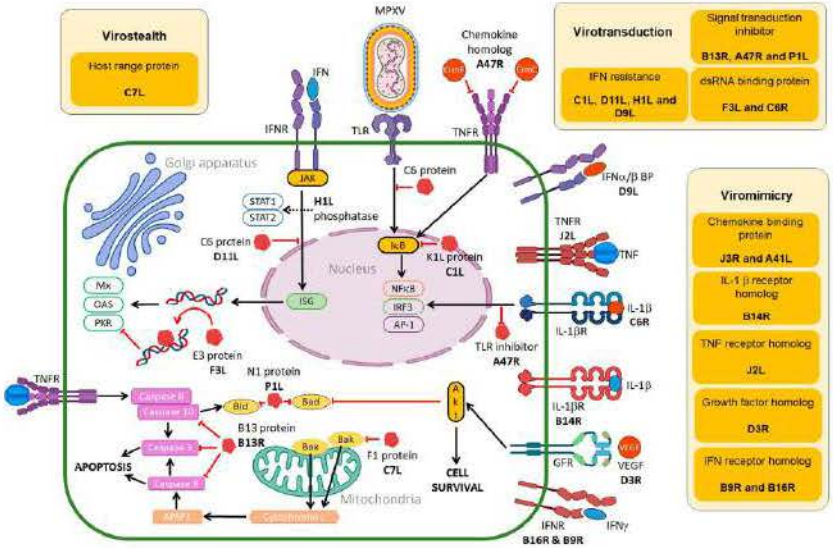

Figure 3. MPXV proteins (red) that participate in virostealth, viromimicry and virotransduction are responsible for immune evasion mechanisms of MPX infection [134–137]. In viromimicry, MPXV mimics host receptors that inhibit binding of IFN, IL-1β and TNF as well as MPXV-encoded chemokines and growth factors. In virotransduction, several antiviral pathways including IFN, NF-κB, IRF3 and apoptosis are interfered with by intracellular MPXV-encoded proteins to inhibit their functions. Virostealth is achieved with F1, an anti-apoptotic host range protein that helps with viral replication and the spread of MPX infection (Created with BioRender.com).

glycoproteins that resemble host immune-related cytokine and chemokine receptors, and bind with them and dysregulate their functions, while virokines mimic host cytokines and chemokines and inhibit their functions [136,137]. MPXV immunomodulatory proteins act synergistically to evade the host antiviral innate immune response through different strategies to allow for viral replication (Figure 3).

Following MPX infection, PRRs such as cytosolic DNA sensors recognize viral DNA and engage defence mechanisms in the host, including production of interferon (IFN) and other pro-inflammatory mediators (IL-6, IL-8, G-CSF) in infected cells and immune cells, and activate the complement cascade [138,139]. Upon MPX infection, the concentration of APCs such as monocytes along with natural killer (NK) cells that directly kill infected cells increases significantly [138,140]. Macrophage-secreted IL-1α and IL-β are elevated in the mild stage of MPX infection, and IL-10, GM-CSF and IL-2R are strongly elevated in the severe stage of infection [141]. Both CD4+ and CD8+ T cells are activated via T cell receptor (TCR) stimulation to recognize APCs and virus-infected cells, respectively. CD8+ T cells are activated on recognition of epitopes derived from F8L protein in MPXV-infected rhesus macaques [142]. In these animals, generation of humoral and cellular immune responses against the virus is characterized by increased levels of B and T cells and production of MPXV-specific IgG and IFN-secreting T cells [143]. These modulations show the immune response to MPX infection, in which pro-inflammatory mediators are secreted to promote migration of immune cells to the site of infection and immunosuppression occurs at the end of aggressive infection through dampened immune responses.

Similarly to other viruses, MPXV has several strategies to evade immune responses. Immunity-related and disease-specific pathways are overexpressed. MPX infection caused by intracellular pathogens or inflammatory processes involves leukocyte chemotaxis or activation of immune cells [84]. MPXV selectively inhibits the expression of genes responsible for cell signalling pathways that activate innate immune responses [144]. IFN is one of the main innate mediators after viral infection, and susceptibility to and severity of the infection are increased when IFN is insufficient [145]. MPXV interferes with IFN signalling pathways through several strategies. It and other poxviruses express a variant form of IFNα/β binding protein, B18, that binds to the cell surface of surrounding uninfected cells and protects cells from the antiviral effects of IFN before cells become infected [146,147]. Studies on VACV have shown that B18

attaches to the cell surface by interaction with glycosaminoglycans, an interaction shared by the IFNα/βBP encoded by MPXV [147,148]. Like VACV, MPXV encodes F3 protein, a homologue of VACV E3 protein that, although truncated, is capable of blocking activation of innate immune cells, thus evading the antiviral IFN system [149]. This truncated E3 protein binds to double-strand RNA (dsRNA) of the virus in infected cells and sequesters it from recognition by PRR, thus inhibiting activation of the protein kinase R (PKR) pathway and supporting viral replication [149]. E3L-specific T cells derived from SPX-vaccinated individuals effectively kill peptide-loaded target cells and VACV-infected cells *in vitro* and the epitopes are shared with MPXV, suggesting E3L as a target protein in vaccine development [150].

In an innate immune response, IL-1β produced by monocytes and macrophages binds to IL-1 receptor and stimulates TNF, IL-2 and other cytokine receptors [151]. A truncated version of BR-209, an IL-1β binding protein, is present in MPXV [49]. BR-209 prevents IL-1β from binding to IL-1 receptors and inhibits the inflammatory cascades. Another host immune defence mechanism, the complement system, is dysregulated by MPXV through genes that encode complement control protein. The MPXV inhibitor of complement enzymes (MOPICE) modulates the antiviral immune response against MPX, as observed by enhanced viral replication *in vivo* and dampened adaptive immune response in a primate model of infection lacking MOPICE expression [139,143]. MOPICE is only expressed in Central African MPXV and is hypothesized as a virulence factor for increased pathogenic properties of this clade compared to the West African clade. Chen et al. [33] compared the sequences of MPXV isolates from West Africa with Congo Basin isolates and identified several possible virulence genes (D10L, D14L, B10R, B14R, B19R) with D14L that encodes MOPICE as the leading candidate. MOPICE inhibits the early steps of the host complement cascade by acting similarly to the mammalian regulators of complement activation (RCA). It mimics the biological activity of complement regulatory proteins that interact with C3b and C4b to inhibit C3 and C5 convertases in the cascades [152]. MPXV also encodes a secreted chemokine binding protein (vCCI), which is abundantly expressed and secreted from MXPV-infected cells. vCCI binds to macrophage inflammatory protein-1 (MIP-1) and inhibits MIP-1-mediated chemotaxis *in-vivo* and *in-vitro* [153].

Although 96% of the MPXV genome is the same as VARV, marked differences in the regions encoding virulence and host range factors have been identified [32]. BR-203, a virulence protein in orthopoxviruses, has a role in avoiding apoptosis of infected lymphocytes. BR-203 is truncated in the West Africa MPXV clade, whereas the full-length gene is found in the Congo Basin MPXV and is speculated to play a role in its higher virulence. Kindrachuk et al. [154] observed that West Africa and Congo Basin MPXV differentially modulate host cell signalling, as portrayed by the differential virulence of the two clades. Congo Basin MPXV selectively downregulates pathways related to apoptosis and cell proliferation, but enhances cell survival compared to the West Africa clade. BR-203 encoding retains MHC-I in the ER and evades the antiviral activity of CD8+ T cells. However, in contrast to interaction with MHC-I, the homologue of BR-203 in MPXV provides immune evasion by inhibiting activation of CD4+ and CD8+ T cells after cognate interaction with infected cells [155]. It is this homologue that is responsible for rendering T cells non-responsive and is identified as MPXV197 [156]. Instead of interfering with antigen presentation or the ability of T cells to respond, MPXV197 directly inhibits T cells through TCR stimulation. Another mechanism is infecting primary human monocytes that are poorly recognized by antiviral CD4+ and CD8+ T cells [155]. However, studies have identified several virulence factors of MPXV that simultaneously regulate host range and immunomodulatory genes in which no individual gene is solely responsible for pathogenicity [157]. Genomic deletion of two particular regions in MPXV effectively inhibits viral replication, tissue spread and mortality *in-vitro* and *in-vivo* with no greater inhibition in either single or dual deletions [157].

Clinical presentations

MPXV incubates for 10–14 days followed by an interval of 1–3 days, during which patients start to suffer from general signs and symptoms of viral infection and the SPX-like skin rash develops [158,159]. MPX disease begins as nonspecific symptoms such as backache, headache, chills, fever, fatigue, myalgia, lethargy and lymph node swelling (Figure 4). After three days, the fever decreases and the rash spreads centrifugally over the body [161,162]. Similarly to SPX rash, it first evolves as macules for 2–4 weeks, and then transforms into papules, vesicles, pustules and finally crusts and scabs [163]. These types of rash can be seen simultaneously during disease progression and last around two to four weeks. The numbers reach up to the thousands, with diameters of 0.5–1 cm and start from the trunk and then spread across the body with a centrifugal pattern of distribution. A centripetal pattern has been reported in a minority of patients [161,162]. Severe lymph node enlargement in the neck, axillary and groin regions are observed and can distinguish MPX from other infections [164]. Onset of rash has been suggested to be the starting point of the infectious period, but the Centers for Disease Control and Prevention (CDC) have stated that

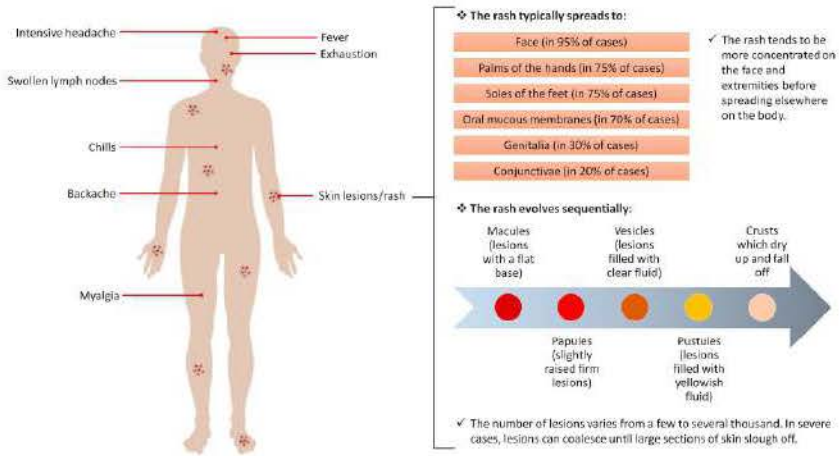

Figure 4. Common symptoms of MPX according to the WHO [160] (Created with BioRender.com).

this period may start before the appearance of the rash during the prodromal symptoms [165,166].

In severe cases, complications may include conjunctivitis, eye damage resulting from corneal infection, diarrhoea and vomiting resulting in dehydration, encephalitis, tonsillitis, pharyngitis and, uncommonly, bronchopneumonia [161,167,168]. Most reported deaths have been in immunosuppressed patients, young adults and children, with a rate of 1–10%. Some changes have occurred regarding the clinical features and complications after the emergence of vaccines. Higher fatality, more robust rash and more severe clinical presentations have been reported in unvaccinated patients [167,169]. The endemic source also relates to the nature of the disease, as African patients show clinical pictures that are similar but more severe than US cases [162].

Given the nonspecific signs and symptoms of MPX, other diseases should be considered during differential diagnosis, including rickettsia, anthrax, syphilis, measles and scabies, SPX and chickenpox (caused by the varicella-zoster virus (VZV)) [170]. For instance, SPX shows more severe clinical manifestation and evolves as a monomorphic rash (vesicles or pustules), unlike MPX, which emerges polymorphically [171–173]. The lesions of chickenpox are smaller and more superficial than MPX and distributed on the trunk rather than the limbs [170,174]. Lymphadenopathy distinguishes MPX from both SPX and chickenpox.

The virulence of MPXV varies based on the origin of the isolates. The Congo Basin clade has the highest virulence [3,33]. The median lethal dose (LD_{50}) of West African MPX is 1.29×10^5, while that of the Congo Basin clade is 5.9×10^3, which was more virulent in the prairie dogs based on morbidity and mortality. Intranasal or intraperitoneal inoculation of adult ground squirrels (*Spermophilus tridecemlineatus*) with $10^{5.1}$ PFU of West African MPXV leads to anorexia and lethargy within four to five days of infection. Inoculation with Congo Basin MPX is associated with acute severe respiratory tract infection and death within a few weeks. The mortality rate of prairie dogs after inoculation with $10^{5.1}$ PFU of West African MPXV varies based on the route of viral administration, with a rate of 100% by intranasal and 60% by intraperitoneal inoculation [64,175,176]. Furthermore, in cases infected with Congo Basin clade, MPX caused more frequent skin lesions and cutaneous eruptions [177].

Transmission modes

Despite the name, MPXV is mainly found in rodents, which are the likely animal reservoir, and this might have contributed to its emergence in humans [178]. However, the natural reservoir of MPXV remains unknown. The human outbreaks in West Africa and elsewhere were transmitted from rodents and other animals due to climate change, rainforest exploitation and highly mobile populations [7]. MPX infection was recognized as a zoonotic disease that infects a wide range of animals, including chimpanzees, lesser and greater white-nosed monkeys, grivets, red colobus monkeys, African brush-tailed porcupines and the Gambian sun squirrel [179,180]. Since MPX is a neglected disease, the pathogenesis in humans is not well studied [181]. MPX is being recognized as an epizootic disease in humans, and is sometimes regarded as a lethal infection [159], with the risk of transmission from human to human [159,182].

There are two possible modes of MPXV transmission: human-to-human and animal-to-human (Figure 5). Human-to-human transmission is possible through direct exposure to respiratory droplets and body fluids from infected patients [65,161,162,183–185]. Thus, MPXV can spread through any form of close contact with someone who is infectious, including sexual contact. Also, a pregnant woman can pass the disease to the fetus during pregnancy, or to the newborn after pregnancy by close contact. Studies have shown nosocomial and sexual transmission of MPXV [65,186–188]. Zoonotic infections occur by direct contact with mucocutaneous lesion content, body fluid and blood of infected animals, or even by consuming undercooked meat of an infected animal [65,161,162,183–185].

Human acquired MPXV in most cases is linked with the handling of infected animal tissue [189]; for example, the aetiology of 91% of cases reported in the 1970s (43 of 47 patients) was direct contact with infected animals in comparison with only 9% (4 of 47) following contact with infected humans [29,182]. Reynolds et al. [190] found a significant correlation between the route of MPXV transmission and clinical manifestation. Complex exposure that recognizes groups of patients who were scratched and bitten by infected animals, in addition to exposure to non-invasive virus transmission such as touching or standing close (within 6 feet) to an infected animal or fomite transmission were significantly associated with serious systemic illness and the need for hospitalization, in comparison to non-invasive exposure [190]. Complex exposure is also associated with a lack of febrile prodromes and short incubation periods (9 days in comparison to 13 days in non-invasive exposure) [190].

The MPXV transmission rate, mortality and route of transmission vary based on the virus strain, with the Congo Basin strain having a high transmissibility rate than the West African strain [159]. Congo Basin isolates are more virulent in humans than those isolated from West Africa [33]. Circulating MPXV strains in West Africa and the US have been reported with no fatalities and no human-to-human transmission [191].

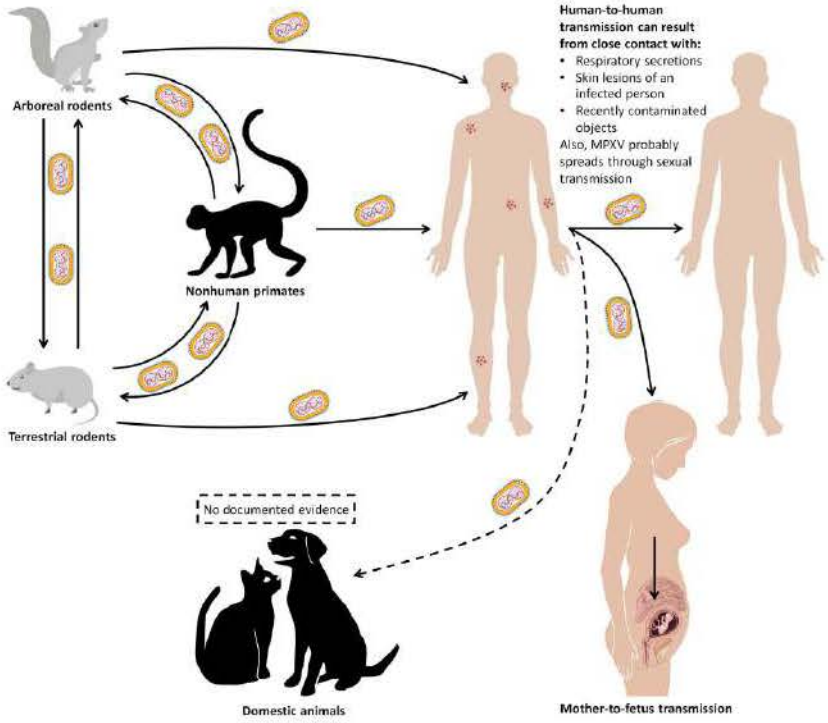

Figure 5. Transmission routes associated with MPXV according to the WHO [160] (Created with BioRender.com).

Epidemiology

In the last century, the incidence of human MPX disease was rare and only sporadic cases were reported in several countries in Africa. The first human case of infection with MPX was identified in 1970s in the DRC and linked to a nine-month-old male child [2,192]. This was followed by other sporadic cases reported in 11 other African countries: Cameroon, DRC, Nigeria, Benin, the Republic of the Congo, the Central African Republic, Gabon, South Sudan, Côte d'Ivoire, Sierra Leone and Liberia [160,193]. A large MPX outbreak was reported in the DRC from February 1996 to February 1997 and involved 511 infected cases [194].

In 2003, an MPX outbreak occurred in the US with 47 confirmed or probable cases. It was thought that the infected patients were exposed to the virus through infected prairie dogs kept in a pet distribution facility with other mammals, including the expected first host, African rodents from Ghana [195]. Petersen et al. [161] reported that 116 confirmed cases with a mortality rate of 6.7% and another 280 suspected cases occurred in Nigeria at the end of 2018, with most cases in people below 40.

The incidence of the disease has increased dramatically and the DRC reported a 20-fold increase in the number cases between 1981–1986 (7.2/100,000 population) and 2006–2007 (144.2/100,000 population) and a 5-fold increase from 2001 (0.64/100,000 population) to 2012 (3.11/100,000 population) [196]. Bunge et al. [197] extracted data from 28 peer-reviewed published articles and 15 grey literature reports on human MPX disease and found that the number of cases has increased since the 1970s, with an increase in the median age of infected cases from 4 years old in the 1970s to 21 years old from 2010 to 2019.

In the previous outbreaks, MPX was reported in children and adolescents in the endemic regions and it was believed that the clinical picture and severity of symptoms are the same as those in adults. However, the WHO has recently reported that severe MPX cases occur more commonly among children and are related to the extent of virus exposure. Furthermore, patient health status, nature of complications and underlying immune deficiencies may be associated with worse MPX outcomes [160]. Adults who were born after the 1980s are at increased risk because vaccination against SPX, which may protect against MPX, ceased after eradication of SPX in the 1980s [7]. Moreover, it was believed that MPX infects males and females equally, but many cases of MPX have occurred in men who have sex with other men (MSM) in the current multi-country outbreak. The CDC reported that MSM make up the majority of MPX cases in the 2022 outbreak, which puts gay, bisexual and transgender people at increased risk of MPX infection [166]. Uncertainty remains on the sexual transmission routes of MPXV between MSM and further studies are needed to better understand this risk.

Coinfection of MPX with other sexually transmitted diseases (STD) and blood borne pathogens has been reported [198], but patients with human immunodeficiency virus (HIV) have the most concern since infection with HIV is considered to be a risk factor for MPX during the current outbreak [199,200]. Lack of appropriate immune response in cases of advanced or uncontrolled HIV infection is significantly associated with a poor prognosis, longer duration of MPX signs, delayed curing of self-limiting MPX infection, other comorbidities, and complicated treatments [198,201]. Therefore, screening of MPX patients for HIV is highly recommended in MSM [198]. Infection with MPX has also recently been recognized as a factor that increases the probability of HIV infection [202–204]. A recent cross-sectional descriptive study from Madrid, Spain found that 44.3% (225/508) of confirmed MPX cases also had HIV infection [201]. Another study from London, UK showed that 35.9% (70/195) of confirmed MPX cases had concomitant HIV infection [205]. Also, mild MPX infections among HIV/AIDS patients have been reported from Portugal and Italy [206,207], especially among individuals with increased T-helper cells count, undetectable HIV viral genetic material, and under anti-retroviral therapy [208]. Patients with immunosuppression caused by HIV had a distinct wide spectrum of clinical manifestations concurrently with typical MPX lesions. Exanthema, fever, genital ulcers and inguinal lymphadenopathy were significant in MPX patients during the ongoing outbreak in Portugal [206]. Papules, pustules, umbilicated with a necrotic central lesion in the perianal area, genitals, mouth, trunk and face were reported in a 24-year-old bisexual man with acute HIV infection [209]. Furthermore, during the 2017–2018 MPX outbreak in Nigeria, more than half of MPX deaths were in patients with uncontrolled HIV with AIDS manifestations who were not receiving antiretroviral therapies [210]. Another study from Nigeria found that HIV-coinfected MPX cases had more prolonged illness, larger lesions, and higher rates of both secondary bacterial skin infections and genital ulcers, compared to HIV-negative MPX cases [211]. Coinfection with another STD was also reported among HIV/MPX patients. A patient with undiagnosed advanced HIV was recently reported with syphilis, and presented with nasal necrosis, severe penis and oral mucosa infections, and MPX lesions distributed over the whole body [212].

Active MPX disease surveillance was conducted in nine health zones in central DRC from November 2005 to November 2007 and 760 laboratory-confirmed cases of MPX were found, with an overall annual incidence of 55.3/100,000 population. Male gender, age

<15, no SPX vaccination and living in forested areas were major risk factors for infection [213]. In 2017, a large outbreak of MPX was reported in Nigeria, with over 500 suspected, over 200 confirmed cases, and a mortality rate of 3% [214]. In another study, Beer and Rao [215] analyzed 71 documents describing MPX cases and outbreaks between 1970 and 2018. The reported outbreaks were found to have increased since 1970, with a total of 35 reported outbreaks outside the DRC, including 20 between 2010 and 2018. Table 3 provides data on human MPX cases and deaths by country from previous outbreaks.

Table 3. Number of MPX cases and deaths from 1970 to 2018.

Country	Time frame	Total suspected cases	Total deaths	References
Democratic Republic of the Congo	1970	1	1	[1]
	1981–1986	338	33	[179]
	1996–1997	773	8	[216]
	2001	388	13	[196]
	2002	881	14	[196]
	2003	755	16	[196]
	2004	1024	29	[196]
	2005	1708	26	[196]
	2006	783	20	[196]
	2007	970	11	[196]
	2008	1599	67	[196]
	2009	1919	27	[196]
	2010	2322	26	[196]
	2011	2208	15	[196]
	2012	2629	34	[196]
	2013	2460	37	[196]
	2016	155	11	[217]
	2019	3794	73	[218]
	2020	4594	171	[218]
Central African Republic	2001	8	2	[219,220]
	2010	2	0	[219]
	2015	3	1	[220]
	2015–2016	62	5	[221,222]
	2017	8	0	[193]
	2018	33	1	[223]
Republic of the Congo	2003	12	1	[224]
	2010	11	1	[225]
	2017	88	6	[193]
Sudan	2005	37	0	[226]
Cameroon	1989	1	0	[227]
	2018	16	0	[223]
Gabon	1987	1	1	[228]
	1991	9	0	[193]
Nigeria	1971	2	0	[177]
	1978	1	0	[177]
	2017–2018	228	6	[65]
Sierra Leone	1970–1971	1	0	[177]
	2014	1	1	[193]
	2017	1	0	[193]
Liberia	1970–1971	4	0	[177]
Côte d'Ivoire	1971	1	0	[229]
USA	2003	47	0	[230,231]
	2021	2	0	[232]
UK	2018	4	0	[233]
	2019	1	0	[234]
	2021	3	0	[235]
Singapore	2019	1	0	[236]

Source: Adopted from Brown and Leggat [162]; Beer and Rao [215]; Adegboye et al. [162,215,237].

On 5 August 2022, the CDC reported 28,220 confirmed cases in 88 countries since 1 January 2022 [238]. Most of these cases (27,875) were reported from 81 countries that have not historically reported MPX [238]. Furthermore, one month ago, the WHO reported several outbreaks of human MPX in regions including the Americas, the Eastern Mediterranean, Europe and the Western Pacific, with a total of 1285 laboratory-confirmed MPX cases. while 1536 suspected and 59 confirmed MPX cases with 72 deaths occurred in Africa from January to June 2022 [232]. Table 4 provides data on global MPX cases and deaths by country from the multi-country 2022 outbreak. Several ecological and environmental factors may have contributed to the emergence or re-emergence of MPX infection, including exploitation of rain forests, climate change, geopolitical and armed conflicts in disease regions, waning herd immunity, highly mobile populations, and the end of SPX vaccination [7,239].

Table 4. MPX cases and deaths reported by the WHO during the multi-country 2022 outbreak (as of 8 June 2022) [232].

WHO Region	Country	Confirmed cases	Suspected cases	Deaths
AFRO	Cameroon	3	28	2
	Central African Republic	8	17	2
	Republic of Congo	2	7	3
	DRC	10	1356	64
	Liberia	0	4	0
	Sierra Leone	0	2	0
	Nigeria	31	110	1
	Ghana	5	12	0
AMRO	Argentina	2	0	0
	Canada	110	0	0
	Mexico	1	0	0
	United States of America	40	0	0
EMRO	United Arab Emirates	13	0	0
	Morocco	1	0	0
EURO	Austria	1	0	0
	Belgium	24	0	0
	Czech Republic	6	0	0
	Denmark	3	0	0
	Finland	3	0	0
	France	66	0	0
	Germany	113	0	0
	Hungary	2	0	0
	Ireland	9	0	0
	Italy	29	0	0
	Israel	2	0	0
	Latvia	2	0	0
	Malta	1	0	0
	Netherlands	54	0	0
	Norway	2	0	0
	Portugal	191	0	0
	Slovenia	6	0	0
	Spain	259	0	0
	Sweden	6	0	0
	Switzerland	12	0	0
	The United Kingdom	321	0	0
WPRO	Australia	6	1	0
Cumulative	36 countries	1344	1537	72

AFRO, Africa; AMRO, Americas; EMRO, Eastern Mediterranean, EURO, Europe; WPRO, Western Pacific; The DRC, Democratic Republic of the Congo

The MPX epidemic threshold is <1, representing the average number of cases caused by an infected person. Thus, MPX infection is likely to be limited to small outbreaks, instead of affecting the whole population. However, the worldwide decline of general orthopoxviral immunity has increased susceptibility to MPX infection [189,213]. In addition, there might be new genetic mutations, leading to widescale outbreaks of MPX [240]. Trend monitoring in 2011–2012 showed that the epidemic threshold of MPX had increased to 1.25 new cases, posing a high risk for health security. Yet, the exact transmission period of MPX, in which it might have been spreading for months or years, remains unknown. MPX has occasionally been endemic in the West and Central Africa areas, with a high number of cases being recently reported. This virus and other orthopoxviruses were commonly controlled through a combined containment strategy [241], and there is reliance on previous and current findings to contain the outbreak.

Laboratory diagnosis

Rapid diagnosis is crucial to eradicating an outbreak, but clinical manifestations are not accurate enough to give a definitive diagnosis. In MPXV-endemic areas with limited resources, a serological test for MPXV-specific antibodies was used before real-time PCR became available [242]. Therefore, the need for diagnostic tools has appeared. Specimens should be taken from skin exudate, vesicular lesions, or crusts and kept cold in a sterile and dry tube. To date, detection of MPXV DNA from extracted nucleic acid using real-time PCR assays is the preferred laboratory method due to its high sensitivity and accuracy [160]. Diagnosis can be confirmed by virus isolation from nasopharynx and oropharynx secretions [161]. Skin biopsies can be obtained from the intact lesion roof or vesiculopustular rash. Certain sera are required in serologic tests to detect the specific immunoglobulin M and G (IgM and IgG) of MPX within 5 and 8 days, respectively [161]. Although this type of testing gives evidence of viral exposure, it also reveals an immune response following vaccination or exposure to other orthopoxviruses [243]. Developing new techniques with more immunological sensitivity could enhance the diagnosis. Some diagnostic tools require large and well-prepared laboratories, but many countries, especially those with the main burdens of the disease, cannot offer these facilities. Accordingly, point-of-care tests are needed without high levels of training suitable for basic laboratories.

Immunohistochemistry (IHC) and histology of common lesions reveal acanthosis, dermal perivascular infiltration, basal vacuolization and keratinocyte necrosis. Spongiosis, ballooning degeneration, epidermal necrosis, viral inclusion, giant cells with neutrophils and eosinophils, and signs of vasculitis are also seen in vesicular lesions. Electron microscopy shows intracytoplasmic structures that are sausage-shaped and oval-to-round inclusions [161].

Haematoxylin and eosin (H & E) stains are used to examine formalin-fixed, paraffin-embedded skin biopsy specimens of MPX infection [244]. Human MPX is histologically characterized by ballooning degeneration of basal keratinocytes and a mild acanthotic spongiotic epidermis that develops into full-thickness skin necrosis of a markedly acanthotic epidermis, containing several viable keratinocytes [245]. The epidermis and superficial dermis are composed of moderate inflammatory infiltrate cells (lymphocytes and neutrophils) with the presence of large multinucleated cells and rare eosinophilic viral inclusion bodies [246]. The keratinocytes exhibit multinucleation with nuclear moulding due to chromatin margination in the epidermis region [230]. In one case report, the papulonecrotic stage of MPX showed early evidence of vesiculation with minimal pustulation. Cell necrosis destroyed the stratum basale layer, while marked hyperplasia and intracellular oedema of stratum spinosum aggregated the papule, leading to formation of spindle cells [244,247]. The rete ridges surrounding the dermal papule were four times deeper with doubled cell layers and an extended area of affected stratum spinosum, in comparison to normal skin. Shallow incomplete stratum granulosum development under the stratum corneum has also been observed [247].

In a novel respiratory model of infection with MPX, the histologic manifestation of the progressing inflammatory lung was correlated with the dose of administered virus. The animal model which survived longest after MPX infection showed distinctive necrotic areas with multifocally fibrin-filled alveoli in the lung, pulmonary fibrosis and oedema, tracheal congestion and fibrous pleural adhesions [248]. The orthopox viral antigen has been detected in degenerating keratinocytes and follicular epithelium of skin biopsy specimens by rabbit anti-VACV polyclonal antibody IHC staining [245]. These findings are supported by the presence of spherical Guarneri intracytoplasmic inclusion bodies located at the affected keratinocytes and their absence in the uninvolved epidermis at the edge of the bullae [230]. A dual IHC staining study of the virus in two animal models showed the presence of abundant viral antigens in most organs and highlighted the colocalization of apoptosis with poxvirus antigen [249]. Both immature and mature stages of assembled virions within the cytoplasm of keratinocytes of glutaraldehyde-fixed skin biopsy human specimens have been observed under transmission electron microscopy. The cross-sections of mature virions have dumbbell-shaped features, and brick-shaped

virions with regularly spaced, threadlike ridges on the exposed surfaces have been viewed on negative-stain electron microscopy (Figure 6) [250]. In general, the lesions of MPX are identical to other viral exanthems such as cowpox virus (CPXV), VARV, VZV, tanapox and herpes simplex virus (HSV) [251].

Current treatment and prevention protocols

During the MPX epidemic in the US in 2003, the CDC stated that taking an SPX vaccine up to two weeks after MPX exposure could reduce the symptoms but not prevent disease [252]. However, the SPX vaccine is neither available to the public nor given to infected patients. This is attributed to concerns over giving a live VACV, its cost and the unknown adverse events among immunocompromised patients [187,252,253]. Patients with low immunity are at high risk of serious side effects from vaccination, including cryptococcal meningitis, cardio-related complications, pneumonia and progressive VACV, which is a rare side effect leading to tissue and skin destruction and can be fatal [254–257]. Second and third generations of SPX vaccines ACAM2000 and Imvamune have been developed, [258] but ACAM2000 has cardiac side effects similar to those reported with the first-generation vaccine [259] and there is no information about its safety among HIV patients [256]. In 2015, the CDC stated that HIV patients and those with a CD4 cell count of 50–199 cells/mm^3 who were exposed to SPX should take Imvamune, while ACAM2000 should be given to those with a CD4 cell count of >200 cells/mm^3. Animal studies have shown that ACAM2000 offers higher viral suppression than Imvamune [256].

There is no specific approved treatment for MPX. Management is limited to treating secondary bacterial infections, reducing the symptoms and giving supportive care [161]. However, there are two drugs, CMX001 and ST-246, developed for the treatment of SPX. ST-246 (tecovirimat) has been approved by the FDA for SPX and has shown efficacy against MPX [260]. Berhanu et al. [261] reported that ACAM2000 alone after MPX exposure was less effective than ST-246 alone or combined with ACAM2000. In a study of the overlapping effects of ACAM2000 and ST-246, the efficacy of the vaccine was largely unaffected, but the humoral response was reduced. Thus, ST-246 should not be given concurrently with ACAM2000 [262]. CMX001 (Brincidofovir) has shown promising results in animal models with various poxviruses

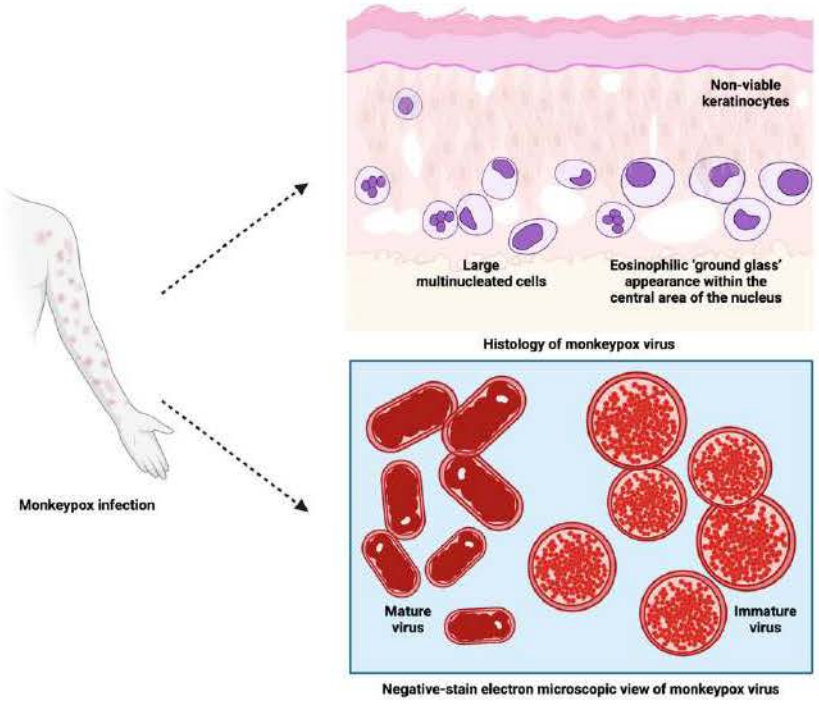

Figure 6. Histology and negative-stain electron microscopic views of MPXV virions (Created with BioRender.com).

Table 5. List of clinical trials of drugs and vaccines against MPX.

Product	Identifier	Type	Developer, Country	Trial location	Status	First Posted
IMVAMUNE®	NCT02977715	SPX vaccine (attenuated live virus)	Bavarian Nordic, Denmark	DRC	Active, not recruiting	30 November 2016
Imvamex®	NCT03745131	SPX vaccine (attenuated live virus)	Bavarian Nordic, Denmark	United Kingdom	Completed	19 November 2018
ST-246®	NCT00728689	Anti-orthopoxvirus compound inhibits release of extracellular virus by targeting the F13L W protein	SIGA Technologies, Inc., United States	United States	Completed	6 August 2008
Tecovirimat (TPOXX, previously ST-246)	NCT02080767	Anti-orthopoxvirus drug that interferes with a p37 viral protein	SIGA Technologies, Inc., United States	-	Available (FDA-approved)	6 March 2014

Data were collected from https://clinicaltrials.gov/.
ST-246®, 4-trifluoromethyl-N-(3,3a,4,4a,5,5a,6,6a-octahydro-1,3-dioxo-4,6-ethenocycloprop [f]isoindol-2(1 H)-yl)-benzamide; DRC, Democratic Republic of the Congo.

including MPX [260]. According to the ClinicalTrials.gov database, as of 19 June 2022, there are four registered trials to evaluate the efficacy of anti-MPX agents (Table 5).

According to the CDC, the following actions help prevent the spread of MPX [166]: avoiding direct contact with individuals who have a rash and flu-like symptoms and appear to be MPX-infected; and avoiding sharing objects and materials (e.g. toilet seats, doorknobs, dishes, bedding, towels or clothing) with someone who has MPX. Also, it is highly recommended to wash hands often with soap and water or use a sanitizer containing alcohol for hand rubbing, especially after using the bathroom or being outside, and before touching the eyes, nose, mouth or face, and before preparing or eating food. Furthermore, people should avoid contact with animals that can get MPX, especially primates and rodents, and even touching their bedding materials. People with probable or confirmed MPX should avoid contact with animals, including pets, to prevent the spread of MPX.

Potential therapeutics

Oral inhibitors of orthopoxvirus infections such as ether lipid prodrugs of cidofovir (CDV) and (S)-HPMPA, ST-246, N-meth-anocarbathymidine (N-MCT) and SRI 21950 (a 4′-thio derivative of iododeoxyuridine) have potentially beneficial effects. HPMPO-DAPy is another high-activity compound that requires parenteral delivery [263].

At doses within a pharmacologically feasible range, CDV, cyclic HPMPC (cHPMPC), HPMPA, ribavirin, tiazofurin, carbocyclic 3-deazaadenosine, 3-deazaneplanocin A and DFBA (1-(2,4-difluorobenzyloxy)adenosine perchlorate), a derivative of adenosine N1-oxide, all inhibited replication of all three VARV strains and the other orthopoxviruses. Two other compounds – methisazone and bis-POM-PMEA – had a weaker antiviral effect. Studies on the sensitivity of 35 strains of VARV and other orthopoxviruses to a subset of three of the most active compounds – CDV, cHPMPC and ribavirin – to examine possible natural drug resistance among VARV isolates obtained from different geographical regions and at different times suggest that nearly all isolates have similar sensitivity [264].

Since 1996, CDV ((S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine, HPMPC) has been approved for clinical use in AIDS patients with cytomegalovirus (CMV) retinitis. CDV is particularly effective against all DNA viruses. VACV, VARV, CPXV, MPX, camelpox (CMPV), molluscum contagiosum and orf (sheep pox) are among the poxviruses [265] that are sensitive to the inhibitory effects of CDV. These findings suggest that CDV may be useful in treatment and short-term prevention of SPX and kindred poxvirus infections [266–268].

Starting antiviral treatment 24 h after lethal intratracheal MPX infection using either the antiviral agent (CDV) or a related acyclic nucleoside phosphonate analogue (HPMPO-DAPy) and different systemic treatment regimens leads to lower mortality and reduced cutaneous MPX lesions. In contrast, no significant reduction in mortality was seen when monkeys were vaccinated 24 h after MPX infection with a standard human dose of a currently recommended SPX vaccine (Elstree-RIVM). All surviving animals had virus-specific blood antibodies and antiviral T cells after antiviral treatment was stopped 13 days after infection. These findings suggest that effective biological threat preparedness should include the ability to treat exposed people with antiviral substances such as CDV or other selective anti-poxvirus medications [269].

The question of how to apply this knowledge to human poxvirus infections remains unresolved. Findings show that the currently recommended CDV dose of 5 mg/kg body weight per week in combination with probenecid (which reduces nephrotoxicity) is unlikely to protect people who have been exposed to VARV infection. It was further shown that the antiviral action is unaffected by the drug delivery schedule. To have a robust protective effect, CDV exposure must be 5–10 times higher than that currently given to patients. However, such high doses may cause nephrotoxicity, and regiments with probenecid administration and dosing schedules that may alleviate CDV uptake into

renal tubular epithelial cells should be considered [270].

When given as early as 5 days before infection or as late as 3 days after with CPXV or VACV (as orthopoxvirus examples), a single dose pre- or post-treatment of mouse models with CDV at 3–100 mg/kg proved successful. Interval treatments with 6.7 or 2 mg of CDV/kg given every third day beginning 72 h after infection were also effective. All mice infected intraperitoneally with ectromelia virus (EV, mousepox) and treated for 7–30 days with CDV died during or after treatment; however, most treated groups experienced significant delays in time to death, and reduced virus proliferation in organs and no CDV resistance was identified [271].

The efficacy of CMX001 (an analogue of CDV; Table 6) as a preventive and early illness antiviral has been tested using rabbitpox virus (RPV) infection of New Zealand white rabbits as a model for SPX. The findings should also apply to MPX infections and the treatment of SPX vaccination side effects [278].

The efficacy of a single dose of CMX001 at 20, 25 or 30 mg/kg doses administered on days 4, 5, 6 and 7 post-infection was examined in A/Ncr mice intranasally infected with modest doses of EV (<20 PFU). To track disease progression, the mice were evaluated for weight loss, blood interferon levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), viral DNA copies and neutrophilia levels. It was discovered that a single dosage of 25 mg/kg of CMX001 given on days 4 or 5 after infection was effective in curing deadly mousepox [277].

ST-246, developed by SIGA Technologies Inc. under licence from ViroPharma Inc., has been found to disrupt a vital phase in morphogenesis of orthopoxviruses. The antiviral activity of ST-246 has been proven in a variety of animal models and its safety was confirmed in healthy human volunteers in a phase I clinical trial [272].

Small compounds like Retro-2 have been shown to reduce orthopox infection *in vitro* and to a lesser extent *in vivo* by inhibiting the retrograde pathway. A vast panel of drugs with a benzodiazepine scaffold-like Retro-1 have been screened to find more effective retrograde pathway inhibitors. When compared to Retro-1, a subset of these compounds had superior anti-VACV activity, resulting in a reduction in extracellular virus (EV) particle formation and viral dissemination [282].

Mycophenolic acid (MPA) and ribavirin, two inhibitors of cellular inosine monophosphate dehydrogenase, have been tested for inhibitory efficacy against orthopoxviruses. 6-aziridine, CDV (HPMPC) and cyclic HPMPC were among the unrelated antipoxvirus drugs studied for comparison. In plaque reduction experiments, MPA suppressed CMPV, CPXV, MPX and VACV by 50% in African green monkey kidney (Vero 76) and mouse 3T3 cells at 0.2–3 μM. Ribavirin was significantly more effective against these viruses in 3T3 cells (50% inhibition at 2–12 μM) than in Vero 76 cells (inhibitory at 30–250 μM) [286]. Table 6 lists the most promising anti-MPX medications, along with evidence for their efficacy, uses, adverse effects, and use in combination therapy.

Vaccines

SPX eradication, managed by the WHO and certified 40 years ago, resulted in most countries discontinuing routine SPX vaccination. Over 70% of the world's population is now thought to be unprotected against SPX and related orthopoxviruses like MPX [7].

In 2018, an outbreak occurred in the UK, but there was little motivation to introduce SPX vaccines to provide cross-protection against MPX [287]. In June 2019, an ad hoc and unofficial group of interested specialists gathered at Chatham House in London to discuss these problems, reviewing available data and identifying MPX-related research needs. It was agreed that a better understanding of the genomic evolution and changing epidemiology of orthopoxviruses, the utility of in-field genomic diagnostics and the best disease control strategies such as vaccination with new generation non-replicating SPX vaccines and treatment with recently developed antivirals were all necessary [7]. Anti-orthopoxvirus IgM and alterations in anti-orthopoxvirus IgG, CD4, CD8, or B-cell responses were found in previously vaccinated MPX cases as indications of a new infection. In MPX cases (vaccinated and unvaccinated), anti-orthopoxvirus IgM and CD8 responses were the most common, with IgG, CD4 and memory B-cell responses indicating vaccine-derived immunity. Immune markers revealed the presence of asymptomatic illnesses in both vaccinated and uninfected people [288].

Active population-based surveillance has been carried out in nine health zones across the DRC. Vaccinated people had a 5.2-fold decreased risk of MPX than those who had not been vaccinated. A comparison of active surveillance data from the 1980s and 2006–2007 in the same health zone showed a 20-fold rise in human MPX incidence. This incidence has risen rapidly in rural DRC 30 years after mass SPX vaccination campaigns ended [213].

Human MPX outbreaks in Africa and the 2003 outbreak in the US have demonstrated that naturally occurring zoonotic orthopoxvirus illnesses remain a public health problem. Vaccination could minimize much of the hazard provided by orthopoxviruses, but because the SPX vaccine is a live orthopoxvirus vaccine, the vaccine can pose a major health risk [289]. Due to a high degree of sequence conservation, vaccinating with VACV also prevents MPX. Antigens

Table 6. Promising anti-MPX medications.

Name	Structure and nature	Evidence	Other Uses and notes	Combined therapy	Side effects
Gidofovir, also called HPMPC or Vistide [269]	[(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, HPMPC] [266] acyclic cytosine phosphonate analogue [272]	Cidofovir has been successfully used in humans to treat persistent molluscum contagiosum and orf in immunocompromised individuals using topical and intravenous routes [266] Targets the viral DNA polymerase and inhibits poxvirus replication in-vitro, ex-vivo and in vivo [272] Effective against almost all DNA viruses and has a broad spectrum of activities [266–268] Starting antiviral treatment 24 h after deadly intratracheal MPX infection using either of the antiviral drugs and a variety of systemic treatment regimens resulted in significantly lower mortality and cutaneous MPX lesions. All surviving animals had virus-specific blood antibodies and antiviral T cells after the antiviral medication was stopped 13 days after infection [269] Three non-human primates were pox-antigen negative after exposure to a fatal dose of MPX and receiving cidofovir. All three primates survived [273]	Resistant orthopox viruses were thought to be untreatable with cidofovir; however, their virulence might be reduced [274] When given as early as 5 days before infection or as late as 3 days after infection with either CPXV or VACV, a single dose pre- or post-treatment with cidofovir at 3–100 mg/kg in mouse models proved successful [271]	Coadministration of cidofovir and Dryvax in a single dosage efficiently minimized vaccination side effects against MPX but severely compromised vaccine-elicited immunological responses and vaccine-induced immunity [275]	Exposure to cidofovir that will have a protective effect against certain orthopox viruses (i.e. VARV major virus) will need doses that are 5–10 times that currently administered to humans, such doses may lead to nephrotoxicity [267]
HPMPA	((S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine) [264]	HPMPA was effective against several orthopoxviruses, with IC50s of 10.9 and 9.2 μg/ml for MPX Vero and LLC-MK2 infected cells, respectively [264] *In vitro*, (S)-HPMPA is active against numerous orthopoxviruses, but not *in vivo*. Ether lipid esters, such as ODE-(S)-HPMPA and HDP-(S)-HPMPA, exhibit better bioavailability and activity against CPXV and VACV infections in mice [276]	HPMPA is closely linked to cidofovir and works similarly to prevent viral replication. Because cidofovir has already been approved for use in the US and is far more likely to be used to treat poxvirus infections, HPMPA is unlikely to be used in US clinical trials [264]		Oral HDP-(S)-HPMPA and ODE-(S)-HPMPA showed no effect. ODE-(S)-HPMPA, given 72 h after CPXV infection or 24 or 48 h after VACV infection, also reduced mortality. Both compounds have the potential to cure human cases of SPX vaccination-related adverse effects [276]
Brincidofovir (CMX001) HDP-cidofovir	Lipophilic nucleotide analogue formed by covalently linking 3-(hexadecyloxy)propan-1-ol to cidofovir (hexadecyloxypropyl ester of cidofovir) [277]	In comparison to CDV, CMX001 has significantly improved efficacy against all dsDNA viruses [278] Antiviral treatment with CMX001 and ST-246 protects mice infected with the murine MPX in a STAT1-deficient C57BL/6 mouse model [279] CMX001 is effective for symptomatic RPV infection (an orthopoxvirus infection) [280]	Orally available [278]	When administered together on the day of infection, CMX001 and ST-246 provide protection [279]	To date, there has been no evidence of nephrotoxicity in healthy volunteers or critically ill transplant patients [278]
Tecovirimat (ST-246) [281]		Tecovirimat is a new antiviral that targets viral p37 protein orthologs to prevent orthopoxviruses from egressing [281] Has antiviral activity in a variety of animal models [272]	Tecovirimat works as an antiviral by suppressing the generation of EVs, which prevents cell-cell and long-distance propagation [282]	ACAM2000 (SPX vaccine) given after exposure did not protect against severe MPX disease or death, while post-exposure treatment with tecovirimat alone or with ACAM2000 gave complete protection. Tecovirimat after infection was 83% (days 4 and 5) or 50% (day 6) effective [261]	Following a phase I clinical investigation, safety was validated in healthy human volunteers [272]

(Continued)

Table 6. Continued.

Name	Structure and nature	Evidence	Other Uses and notes	Combined therapy	Side effects
Retro-2 and analogues		PA104 was the most effective Retro-2 analogue, suppressing viral propagation by 90% at 1.3 M with a good selectivity index. These findings, and additional identification of PA104's specific protein targets and *in-vivo* activity, could be significant for development of effective antivirals for OPXV [282]			PA104 inhibited two different ST-246-resistant viruses, indicating that it could be useful in combination therapy with ST-246 [282]
siRNAs		At a dose of 1 nM, siRNAs siB1R-2 and siG7L-1 reduced MPXV viral proliferation by 95%. Without inducing a beta interferon response, siB1R-2 and siG7L-1 silenced their corresponding transcript B1R and G7L mRNAs [283] Seven siRNA constructs suppressed viral replication in cell culture by 65-95% with no apparent cytotoxicity, targeting either an essential gene for viral replication (A6R) or a key gene in viral entry (E8L). Further tests using wild-type and recombinant MPX producing green fluorescent protein revealed that siA6-a was the most powerful construct, inhibiting viral replication for up to 7 days at a dose of 10 nM [284] Different pathogenic orthopoxviruses (CPXV and MPXV) were inhibited up to 70% at the lowest concentration (1 nM) tested, indicating siD5R-2 (siRNA targeting D5 protein) efficacy. siD5R-2 had antiviral effects in human keratinocyte and fibroblast cell cultures infected with VACV [285]		When siB1R-2, siG7L-1, or siD5R-2 were coupled with cidofovir, strong synergistic effects were seen. Combination therapy of siRNA and cidofovir could be effective in treating poxvirus infections [283]	
Mycophenolic acid (MPA)		In plaque reduction experiments, MPA suppressed CMPV, CPXV, MPXV and VACV by 50% in African green monkey kidney (Vero 76) and mouse 3T3 cells at 0.2–3 μM [286]	Anti-orthopoxvirus efficacy of ribavirin is boosted by other modes of virus inhibition. Biological variations in mode of action and the immunosuppressive potential of ribavirin and MPA, cause the former to be effective against orthopoxvirus infections in animals, while the latter is not [286]		MPA and ribavirin were more toxic to replicating cells than stationary cell monolayers, with greater toxicity in 3T3 cells than Vero 76 cells. Compared to Vero 76 cells, the higher antiviral efficacy and toxicity of ribavirin were due to more accumulation of mono-, di- and triphosphate forms of the drug in 3T3 cells. Virus inhibition was linked to suppression of intracellular guanosine triphosphate pools for MPA and ribavirin [286]
Ribavirin		Ribavirin was significantly more effective against these viruses in 3T3 cells (IC50 2–12 μM) than in Vero 76 cells (IC50 30–250 μM) [286]			

Table 7. Vaccines of various sorts and their potential applications in the prevention and treatment of MPX.

Vaccine	Efficacy against MPX	Notes
First-generation SPX vaccines: Dryvax and five VACV strains like Lister, Copenhagen (Cop), chorioallantois VACV Ankara (CVA), Tian Tan (TT), Bern and New York City Board of Health (NYCBH)	– Dryvax (freeze-dried vaccine) protects against SPX and MPX [292] – Due to a high degree of sequence conservation, vaccinating against SPX with VACV may protects against MPX [290]	– Associated with serious complications in both naïve and immune people [293]. First-generation SPX vaccines were used in the eradication program. These VACV strains caused varying levels of vaccine-related complications and as a result, strains like Bern and Copenhagen were favoured less than Lister and NYCBH [294] – Coadministration of cidofovir and Dryvax in a single dose greatly reduced adverse effects, but severely compromised vaccine-elicited immunological responses and vaccine-induced immunity to MPX [275] – Contraindicated in immunocompromised people [292]
VACV vaccine strain LC16m8	– Although lesions were smaller than those induced by the original Lister strain, LC16m8 is less attenuated than MVA and retains the potential to multiply and cause lesions in human vaccinees [295] – Due to a frameshift mutation, LC16m8 does not express B5R protein [296]. As a result, a tiny plaque phenotype develops. Humans immunized with LC16m8 have inadequate immune responses to this protein [297], which is a key target for antibodies that neutralize the EEV form of VACV [298] – Efficacy was compared to that of the original Lister strain. Monkeys were immunized with LC16m8 or Lister and subsequently infected with MPXV strain Liberia or Zr-599 intranasally or subcutaneously. With intranasal-inoculation, immunized monkeys had no symptoms of MPX, but nonimmunized controls showed normal symptoms. With subcutaneous injection, monkeys immunized with LC16m8 showed no signs of MPX except for a small ulcer at the site of MPXV inoculation, while nonimmunized controls showed fatal and typical symptoms. These findings imply that LC16m8 protects monkeys from deadly MPX and may elicit protective immunity against SPX [299]	– Derived from Lister strain in Japan [294] and harbours a mutation in the critical membrane protein B5R [299]
Modified VACV Ankara (MVA); also known as Imvanex in the European Union, Imvamune in Canada, and Jynneos in the United States [300]	– In a STAT1-deficient C57BL/6 mouse model of MPX, vaccination with MVA followed by a booster vaccine protect against an intranasal MPX challenge and elicits a more robust immune response than a single vaccination [279] – In humans and immunocompromised animals, it is safe. The MVA-based SPX vaccine protected macaques against a deadly respiratory challenge of MPX, making it a promising contender for human protection [293] – To produce immune responses comparable to those induced by the initial SPX vaccines, higher viral titres and multiple doses are required [301–303] – Before MPX viral challenge, there was no significant difference in neutralizing antibody levels in animals vaccinated with a single ACAM2000 immunization (132 U/ml) vs. a prime-boost Imvamune regimen (69 U/ml) [304]	– A highly attenuated replication-deficient strain of VV [293] derived from strain CVA by Mayr and colleagues in Germany [305] – Obtained after passages of the CVA strain in chicken embryo fibroblasts (CEFs), roughly 15% of its genome was lost due to six big deletions and many smaller alterations [306,307]. MVA is unable to reproduce in most mammalian cells in culture, including human cells and lacks several immunomodulators and host range genes seen in other VACV strains [308,309] – The prime-boost Imvamune group had evidence of viral excretion from the throats of two of six animals after challenge [304]
New York VACV (NYVAC)	NYVAC, like MVA, is unable to replicate in human cells. However, unlike MVA, expression of several late NYVAC proteins is restricted due to a translational block [310]. Despite being attenuated, NYVAC still elicits a strong immunological response [311,312]	Derived from a plaque-cloned isolate of the Copenhagen vaccine strain by deletion of 18 ORFs from the viral genome. Among the deleted ORFs, two genes are involved in nucleotide metabolism, the thymidine kinase (OFP J2R) and the large subunit of the ribonucleotide reductase (ORF I4L) [313]
DNA vaccine consisting of four VACV genes (L1R, A27L, A33R and B5R)	– After an otherwise deadly challenge with MPX, rhesus macaques treated with a DNA vaccine were protected from severe illness. Vaccinated animals with a single gene (L1R) that encodes a neutralizing antibody target developed	

(*Continued*)

Table 7. Continued.

Vaccine	Efficacy against MPX	Notes
	severe illness but survived. This is the first proof that vaccinating against SPX and MPX with a subunit vaccine is possible [289] – Animals given only DNA did not have high titre Abs, had numerous skin lesions after being challenged and died similarly to placebo controls. Animals given proteins had moderate to severe illness (20–155 skin lesions) but lived. Individuals inoculated with DNA and then boosted with proteins had minimal illness, with 15 or fewer lesions that resolved in a matter of days [292]	
Dryvax-derived ACAM2000 (second-generation smallpox vaccines)	– ACAM2000 did not provide protection against severe MPX disease or mortality when given after exposure [261] – Prairie dogs were protected to some extent from the 2 LD 50 challenge of MPX disease, but not from the 170 LD 5 challenge. In the 2 LD 50 challenge, giving the vaccine one day after exposure was more effective than giving it three days later for Imvamune, while ACAM2000 was equally efficacious at both post-exposure vaccination times [314]	– Treatment with tecovirimat alone or in combination with ACAM2000 after exposure gave complete protection [261] – The only SPX vaccine now available in the United States that has been licensed by the FDA [294]
– Elstree-RIVM and Elstree-BN	No substantial reduction in mortality was seen in monkeys vaccinated 24 h after MPX infection with a typical human dose of Elstree-RIVM. All surviving animals had virus-specific blood antibodies and antiviral T cells after antiviral medication was stopped 13 days after infection [269]	– Elstree-RIVM is a first-generation SPX vaccine produced on calf skins [293] – Elstree-BN is a second-generation vaccine, passaged and produced on CEFs to further attenuate the virus and to make a better-defined vaccine preparation that does not depend on the use of calves [293]

within the MPX proteome that contribute to immune responses have yet to be identified in detail [290]. In the past, people who had been exposed to SPX were treated with the SPX vaccine and VACV immune globulin (VIG). Patients who were at high risk of problems following SPX immunization were also given VIG. As a result, post-exposure vaccination and VIG therapies may become again essential therapeutic options [291].

A protein microarray was used to capture antibody responses to MPX infection and human SPX vaccination. Only 14 of these proteins were recognized by IgG from vaccinated humans, but serum IgG from cynomolgus macaques recovering from MPX recognized at least 23 proteins within the orthopox proteome. Twelve of the 14 antigens discovered by human vaccines were also recognized by convalescent macaque IgG. The structural proteins F13L and A33R and the membrane scaffold protein D13L had the highest level of IgG binding. Before onset of clinical symptoms, significant IgM responses to the MPXV's A44R, F13L and A33R were observed. Antibodies from vaccination recognized a limited number of proteins shared with pathogenic virus strains, although humoral responses to antigens specific to the MPXV proteome were also required for recovery from infection [290]. Different types of vaccines and their potential uses in prevention and treatment of MPX are shown in Table 7.

Conclusions

Epidemiological research to control the current MPX outbreak should consider the source of infection and all transmission routes. The current therapeutic regimens and vaccines that have been shown to be effective against SPX offer new approaches to the clinical treatment and prevention of MPX, which is essential in control of the current outbreak. Although management of MPX infection is still limited to treating secondary bacterial infections, reducing the symptoms and giving supportive care, FDA-approved anti-SPX treatments (CMX001 and ST-246) have shown efficacy against MPX. In this emergency situation, testing treatments with proven antiviral activities against VARV or other poxviruses may promote the pace of development of anti-MPXV drugs. Moreover, the world should turn the obstacles faced during outbreaks of infectious diseases that have emerged in the past into lessons to control the current outbreak by active cooperation, which is important in global efforts to combat the outbreak.

Acknowledgements

M.A.I. Al-Hatamleh would like to acknowledge the Universiti Sains Malaysia (USM) Fellowship Scheme for providing financial support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by a grant from the Ministry of Higher Education, Malaysia (Grant number: FRGS/1/2020/SKK06/USM/03/2).

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AKTA HAK CIPTA 1987

PERATURAN-PERATURAN HAK CIPTA (PEMBERITAHUAN SUKARELA) 2012

SIJIL PEMBERITAHUAN HAK CIPTA
[Subperaturan 8(2)]

No. Pemberitahuan	:	**CRLY2023P00622**
Tajuk Karya	:	**TUBERKULOSIS PENYAKIT TIBI PARU-PARU DAN EKSTRAPULMONARI TIBI**
Kategori Karya	:	**SASTERA**
Tarikh Permohonan	:	**22 FEBRUARI 2023**

Saya dengan ini mengesahkan di bawah Akta Hak Cipta 1987 [Akta 332] dan Peraturan-Peraturan Hak Cipta (Pemberitahuan Sukarela) 2012 bahawa karya hak cipta dengan No. Pemberitahuan seperti di atas bagi pemohon **UNIVERSITI SAINS MALAYSIA** sebagai **PEMUNYA** dan **RAMLAH BINTI KADIR (801106115392), ROHIMAH MOHAMUD (810107035954), NORHAYATI BINTI YUSOP (830809145670), NOR EFFA SYAZULI BINTI ZULKAFLI (830806065034), NURFATIHAH AZLYNA BINTI AHMAD SUHAIMI (950111105526), NOR ASYIKIN BINTI NORDIN (961007036034)** sebagai **PENCIPTA** telah didaftarkan ke dalam Daftar Hak Cipta menurut seksyen 26B Akta Hak Cipta 1987 [Akta 332].

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KAMAL BIN KORMIN
TIMBALAN PENGAWAL HAK CIPTA
MALAYSIA

(Agensi di bawah Kementerian Perdagangan Dalam Negeri dan Kos Sara Hidup)

4 PENYAKIT TIBI UROGENITAL

Jangkitan berlaku apabila kuman tibi menyerang sistem pembiakan serta salur kencing

Simptom:

- Pembengkakan testis
- Nyeri ketika buang air kecil
- Kencing berdarah
- Aliran urin yang tidak lancar/sedikit
- Nyeri di bahagian pinggul
- Nyeri pada tulang belakang
- Isipadu air mani berkurang
- Ketidaksuburan

DIAGNOSIS TIBI:

UJIAN MANTOUX

UJIAN MAKMAL:

1. PEMERIKSAAN KAHAK
2. KULTUR BAKTETI 'ACID FAST BACILLI' (AFB)
3. PEMERIKSAAN X-RAY

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5 PENYAKIT TIBI KELENJAR LIMFA

Jangkitan berlaku apabila kuman tibi berpindah ke kelenjar limfa terdekat, seperti di leher, ketiak dan pangkal paha.

Simptom:

- Benjolan pada leher, ketiak dan pangkal paha
- Limfa membengkak dan nyeri
- Sukar bernafas
- Batuk
- Berpeluh di tengah malam

RAWATAN:

KAEDAH: DIRECTLY OBSERVED TREATMENT (DOTS)

UBAT-UBATAN: STREPTOMYCIN, ISONIAZID, RIFAMPICIN, PYRAZINAMIDE, ETHAMBUTOL

CARA PENCEGAHAN:

PENGAMBILAN VAKSIN: BACILLUS CALMETTE-GUÉRIN (BCG)

Hakcipta terpelihara @2022

Program KPT Prihatin Komuniti Sejahtera (KRIS)

Dr. Ramlah Kadir, Nor Asyikin Nordin, Norfatihah Azlyna Suhaimi, Dr. Norhayati Yusop, Dr. RohimahMohamud, Dr. Nor Effa Syazuli Zulkafli

KEMENTERIAN PENGAJIAN TINGGI

USM UNIVERSITI SAINS MALAYSIA

APEX

TUBERKULOSIS

PENYAKIT TIBI PARU-PARU DAN EKSTRAPULMONARI

STATISTIK KES TB

SELURUH DUNIA:
2019 & 2020:
~10 JUTA KES

MALAYSIA:
2019: 26,352 KES
2020: 23, 644 KES
2021: 21,727

Jabatan Immunologi
Pusat Pengajian Sains Perubatan

2 PENYAKIT TIBI TULANG

Jangkitan berlaku apabila berlakunya pengaktifan semula kuman tibi yang tertanam dalam bahagian tulang belakang atau sendi besar

Simptom:

- **Timbul benjolan pada tulang belakang**
- **Sakit/tidak selesa pada bahagian tulang belakang**
- **Kebas tangan/kaki atau kedua-duanya**
- **Lemah/hilang kekuatan tangan atau kaki atau kedua-duanya**

3 PENYAKIT TIBI GESTROINTESTINAL

Jangkitan berlaku apabila kuman tibi yang membiak di dalam paru-paru memasuki saluran gastrousus

Simptom:

- **Nyeri di bahagian perut**
- **Sembelit/ cirit-birit**
- **Mual dan muntah**
- **Perut terasa berat**

PENYAKIT TIBI EKSTRAPULMONARI

- **Jangkitan kuman tibi pada organ-organ lain selain peparu, seperti nodus limfa, pleura, neurologi, sinovial, perikardial, perut, genitouriner**
- **Pada umumnya ia mempunyai simptom seperti tibi paru-paru.**

1 PENYAKIT TIBI MENINGITIS

Jangkitan berlaku pabila kuman tibi yang membiak ke lapisan pelindung otak dan menyebabkan pembentukan abses kecil pada tisu otak.

Simptom:

- **Cepat penat**
- **Hilang selera makan**
- **Sakit kepala yang berterusan**
- **Mual dan muntah**
- **Rasa nyeri di seluruh tubuh**
- **Sensitif terhadap cahaya**
- **Leher terasa kaku**

PENYAKIT TIBI PARU-PARU

- **Jangkitan berlaku apabila titisan air yang mengandungi kuman tibi memasuki saluran pernafasan dan seterusnya ia memasuki paru-paru**
- **Jenis penyakit tibi yang paling kerap berlaku**
- **Kuman tibi merebak di dalam paru-paru menyebabkan kerosakan pada paru-paru**

Penyakit yang disebabkan oleh *Mycobacterium tuberculosis*

Penyakit berjangkit yang tertua dunia

Pembunuh utama manusia selepas penyakit HIV/AIDS

AKTA HAK CIPTA 1987

PERATURAN-PERATURAN HAK CIPTA (PEMBERITAHUAN SUKARELA) 2012

SIJIL PEMBERITAHUAN HAK CIPTA
[Subperaturan 8(2)]

No. Pemberitahuan	:	**CRLY2023P00623**
Tajuk Karya	:	**TUBERKULOSIS DIAGNOSIS MAKMAL**
Kategori Karya	:	**SASTERA**
Tarikh Permohonan	:	**22 FEBRUARI 2023**

Saya dengan ini mengesahkan di bawah Akta Hak Cipta 1987 [Akta 332] dan Peraturan-Peraturan Hak Cipta (Pemberitahuan Sukarela) 2012 bahawa karya hak cipta dengan No. Pemberitahuan seperti di atas bagi pemohon **UNIVERSITI SAINS MALAYSIA** sebagai **PEMUNYA** dan **RAMLAH BINTI KADIR (801106115392), ROHIMAH MOHAMUD (810107035954), NORHAYATI BINTI YUSOP (830809145670), NOR EFFA SYAZULI BINTI ZULKAFLI (830806065034), NURFATIHAH AZLYNA BINTI AHMAD SUHAIMI (950111105526), NOR ASYIKIN BINTI NORDIN (961007036034)** sebagai **PENCIPTA** telah didaftarkan ke dalam Daftar Hak Cipta menurut seksyen 26B Akta Hak Cipta 1987 [Akta 332].

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KAMAL BIN KORMIN
TIMBALAN PENGAWAL HAK CIPTA
MALAYSIA

(Agensi di bawah Kementerian Perdagangan Dalam Negeri dan Kos Sara Hidup)

"Ujian Identifikasi"

Tujuan ujian:

- Mengenalpasti jenis bakteria yang telah berjaya dikultur samada jenis *Mycobacterium tuberculosis* (MTB) atau bukan *Mycobacterium tuberculosis* (non-MTB).

Kaedah yang digunakan:

1. Teknik Konvensional (Pewarnaan pigmen dan ujian biokimia)
2. Ujian Kromatografi Imuno (Immuno Chromatographic Assay, ICA)- teknik paling pantas
3. Ujian amplifikasi asid nukliek (Nucleic Acid Amplification Test, NAAT)

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Universiti Sains Malaysia,
Kampus Kesihatan,
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"Drug Susceptibility Testing"

Tujuan ujian:

- Memberikan panduan dalam pemilihan jenis ubat yang bersesuaian
- Mengenalpasti kemungkinan berlakunya kes dimana pesakit TB telah rintang/imun terhadap ubat yang diberikan semasa rawatan

Kaedah yang digunakan:

1.Kaedah fenotipik- Pengkulturan *Mycobacterium tuberculosis* bagi melihat pertumbuhannya di dalam media yang mengandungi ubatan anti TB

2. Kaedah genotipik- mengesan mutasi gen tertentu yang dikaitkan dengan kerintangan terhadap ubatan TB

Hakcipta terpelihara @2022

Program KPT Prihatin Komuniti Sejahtera (KRIS)
Dr. Ramlah Kadir, Nor Asyikin Nordin, Norfatihah Azlyna Suhaimi, Dr. Norhayati Yusop, Dr. RohimahMohamud, Dr. Nor Effa Syazuli Zulkafli

KEMENTERIAN PENGAJIAN TINGGI

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APEX

TUBERKULOSIS

DIAGNOSIS MAKMAL

Cepat, Mudah, Selamat

Diagnosis makmal tuberkulosis (tibi) melibatkan demonstrasi *Mycobacterium tuberculosis* dengan kaedah mikrobiologi, sitopatologi atau histopatologi

Jabatan Immunologi
Pusat Pengajian Sains Perubatan

"Culture Based Methods"

Tujuan ujian:

1. Mengenalpasti jenis bakteria yang hidup
2. Memperbanyakkan bilangan bakteria
3. Memberikan diagnosis tibi yang tepat

Ujian menggunakan 2 jenis media:

1. Media pepejal- dijalankan secara manual: Loweinsten Jensen (LJ) dan Ogawa
1. Media cecair- menggunakan mesin dan dapat mengesan pertumbuhan kuman secara automatik: Bactec MGIT 960

"LAKUKAN UJIAN DAN RAWATAN SECEPAT YANG MUNGKIN SEBELUM IA MELARAT"

Direct Smear Microscopy

Tujuan:
Mengesanan kehadiran kuman tibi

Ujian berasaskan kepada sifat dinding sel bakteria yang tebal

Kandungan lipid dapat mengekalkan warna walaupun dilunturkan dengan bahan berasid

Kaedah yang digunakan ialah pencelupan Ziehl Neelsen atau Auramine O

SAMPEL UJIAN

Kes TB pulmonari: Sputum atau kahak
Kes TB extrapulmonari/kanak-kanak:
Darah, tisu, cecair badan dan sebagainya

UJIAN BAGI MENGESAN DAN MENGENALPASTI JANGKITAN TB DI DALAM MAKMAL

"Direct Smear Microscopy"
Pengesanan kehadiran *Mycobacterium tuberculosis* menerusi ujian Smear Microscopic

"Culture Based Methods"
Pengesanan pertumbuhan/pembiakan bakteria menerusi ujian kultur media

"Ujian Identifikasi"
Pengenalpastian bakteria menerusi ujian identifikasi

"Drug Susceptibility Testing"
Pengenalpastian kerentanan bakteria menerusi ujian kerentanan antibiotik

AKTA HAK CIPTA 1987

PERATURAN-PERATURAN HAK CIPTA (PEMBERITAHUAN SUKARELA) 2012

SIJIL PEMBERITAHUAN HAK CIPTA
[Subperaturan 8(2)]

No. Pemberitahuan	:	**CRLY2023P00624**
Tajuk Karya	:	**TUBERKULOSIS**
Kategori Karya	:	**SASTERA**
Tarikh Permohonan	:	**22 FEBRUARI 2023**

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KAMAL BIN KORMIN
TIMBALAN PENGAWAL HAK CIPTA
MALAYSIA

(Agensi di bawah Kementerian Perdagangan Dalam Negeri dan Kos Sara Hidup)

FAKTOR PENYAKIT TIBI SUKAR DITANGANI KERANA

1) Sejumlah besar pendatang berasal dari negara yang mempunyai kes tibi yang tinggi

2) Munculnya strain tibi yang rintang ubat

3) Keadaan sosial yang tidak kondusif termasuk kemiskinan, kehilangan tempat tinggal, penyalahgunaan bahan dan kemerosotan infrastruktur penjagaan kesihatan

4) Kepatuhan yang lemah terhadap rawatan tibi

5) Kualiti infrastruktur kawalan tibi yang lemah di sesebuah kawasan

6) Kesukaran dalam komunikasi di antara pesakit dan pengurus penjagaan kesihatan

HUBUNGI KAMI

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Kampus Kesihatan,
16150, Kubang Kerian, Kelantan.

7) Pandangan buruk masyarakat terhadap pesakit tibi dan keluarga pesakit

8) Kualiti persekitaran sosial dan ekonomi seseorang individu yang mempengaruhi semua pencegahan dan kawalan tibi

UBAT ANTI-TIBI:

STREPTOMYCIN
ISONIAZID
RIFAMPICIN
PYRAZINAMIDE
ETHAMBUTOL

VAKSIN TIBI:

VAKSIN BACILLUS CALMETTE—GUÉRIN (BCG)

Hakcipta terpelihara @2022

Program KPT Prihatin Komuniti Sejahtera (KRIS)

Dr. Ramlah Kadir, Nor Asyikin Nordin, Norfatihah Azlyna Suhaimi, Dr. Norhayati Yusop, Dr. RohimahMohamud, Dr. Nor Effa Syazuli Zulkafli

TUBERKULOSIS (TIBI)

Penyakit berjangkit saluran pernafasan disebabkan oleh *Mycobacterium tuberculosis* yang dibawa dalam titisan melalui udara

Jabtan Imunologi
Pusat Pengajian Sains Kesihatan

LANGKAH YANG PERLU DILAKUKAN JIKA DISAHKAN MENGHIDAP PENYAKIT TIBI

1) Pengesanan dan melengkapkan perawatan khusus yang harus dijalani
-Ujian Mantoux
-Ujian darah
-Pemeriksaan kahak, AFB Smear
-X-Ray

2) Atau lakukan ujian diagnostik yang lebih pantas dan sensitif seperti Xpert MTB/Rif

3) Makan ubat secara teratur

4) Tingkatkan sistem imun

5) Tingkatkan nutrisi

6) Elakkan perhimpunan ramai

7) Elakkan bergilir peralatan makanan dan minuman

8) Meningkatkan kualiti penjagaan kebersihan

9) Meningkatkan kualiti sistem pengudaraan di kawasan kemudahan penjagaan kesihatan, pengangkutan awam, tempat kerja dan sekolah

10) Tidur secara berasingan dengan ahli keluarga lain

MANFAAT PENYELIAAN DAN PENJAGAAN INTENSIF

1) Memberi penjelasan yang lebih mendalam tentang penyakit tibi dan sokongan emosi kepada pesakit

2) Meningkatkan kualiti hidup dari segi tempat tinggal, pengangkutan, makanan yang sesuai, sumber keewangan

"LANGKAH PERTAMA DAN PENCEGAHAN YANG CEPAT LAGI EFEKTIF MAMPU MEYELAMATKAN NYAWA ANDA DAN ORANG LAIN"

CARA MENCEGAH PENYAKIT TIBI

1) Menutup mulut ketika batuk dan bersin

2) Sentiasa melakukan disinfeksi

3) Membina imunisasi badan dengan mengamalkan diet pemakanan yang sihat dan seimbang

4) Elakkan meludah di merata-rata tempat

5) Melakukan pemeriksaan dan rawatan awal

6) Mendapatkan sinar matahari yang cukup

7) Tempat tinggal dan persekitaran yang bersih

8) Menghindari kontak langsung

9) Mendapatkan pengetahuan berkaitan dengan penyakit tibi

10) Rehat yang cukup

11) Rajin beriadah/ bersukan

12) Elakkan daripada merokok

AKTA HAK CIPTA 1987

PERATURAN-PERATURAN HAK CIPTA (PEMBERITAHUAN SUKARELA) 2012

SIJIL PEMBERITAHUAN HAK CIPTA
[Subperaturan 8(2)]

No. Pemberitahuan	:	**CRLY2023P00625**
Tajuk Karya	:	**TUBERKULOSIS TIBI DAN JANGKITAN BUKAN TIBI**
Kategori Karya	:	**SASTERA**
Tarikh Permohonan	:	**22 FEBRUARI 2023**

Saya dengan ini mengesahkan di bawah Akta Hak Cipta 1987 [Akta 332] dan Peraturan-Peraturan Hak Cipta (Pemberitahuan Sukarela) 2012 bahawa karya hak cipta dengan No. Pemberitahuan seperti di atas bagi pemohon **UNIVERSITI SAINS MALAYSIA** sebagai **PEMUNYA** dan **RAMLAH BINTI KADIR (801106115392), ROHIMAH MOHAMUD (810107035954), NORHAYATI BINTI YUSOP (830809145670), NOR EFFA SYAZULI BINTI ZULKAFLI (830806065034), NURFATIHAH AZLYNA BINTI AHMAD SUHAIMI (950111105526), NOR ASYIKIN BINTI NORDIN (961007036034)** sebagai **PENCIPTA** telah didaftarkan ke dalam Daftar Hak Cipta menurut seksyen 26B Akta Hak Cipta 1987 [Akta 332].

KAMAL BIN KORMIN
TIMBALAN PENGAWAL HAK CIPTA
MALAYSIA

(Agensi di bawah Kementerian Perdagangan Dalam Negeri dan Kos Sara Hidup)

Penyakit Tibi

Penyebaran melalui udara (Mycobacterium Tuberculosis)

Peyakit berjangkit kedua tertinggi di Malaysia selepas penyakit tangan, kaki dan mulut (HFMD)

Penyakit Bukan Tibi

Bakteria bukan tibi yang menyebabkan jangkitan paru-paru dan bukan tibi

Memberi kesan terutamanya kepada individu:

- ***Mempunyai paru-paru yang telah rosak***
- ***Mempunyai masalah dengan sistem imun***

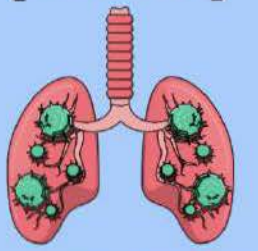
Hubungi kami

09-7676142

Jabatan Immunologi,
Pusat Pengajian Sains Perubatan,
Universiti Sains Malaysia,
Kampus Kesihatan,
16150, Kubang Kerian, Kelantan.

STATISTIK

PEERTUBUHAN KESIHATAN SEDUNIA (WHO) (2020)



9.9 JUTA KES

1.5 JUTA KEMATIAN

1:10

(1 PESAKIT TIBI AKTIF BOLEH MENJANGKITI 10 ORANG SIHAT)

Hakcipta terpelihara @2022

Program KPT Prihatin Komuniti Sejahtera (KRIS)

Dr. Ramlah Kadir, Nor Asyikin Nordin, Norfatihah Azlyna Suhaimi, Dr. Norhayati Yusop, Dr. RohimahMohamud, Dr. Nor Effa Syazuli Zulkafli

Jabatan Immunologi
Pusat Pengajian Sains Perubatan

TUBERKULOSIS (Tibi)

Tibi dan jangkitan bukan Tibi

PARU-PARU

OTAK

BUAH PINGGANG

JANTUNG

TULANG BELAKANG

KELENJAR

	FIBROSIS PARU-PARU	TIBI	PNEUMONIA	INFLUENZA	COVID-19
PENYAKIT PERNAFASAN					
GOLONGAN BERISIKO					
GEJALA					
TEMPOH JANGKITAN	Bergantung kepada ejen penyebab (seperti mikroorganisma berbahaya dan penyakit aktif yang sedia ada)			Sekitar 1-5 hari	5-14 hari
SAMPEL DIUJI	Darah, tisu paru-paru	Kahak, darah		Calitan nasofarinks, air liur	
UJIAN DILAKUKAN	Sinar-X dada, CT	Sinar-X dada, kultur		Kit ujian pantas kendiri, rtk-antigen, rt-pcr	
RAWATAN	Ubat, terapi oksigen	Ubat anti-tibi, BCG vaksin (bayi)	Pneumonia (Vaksin Hib, pneumokokkus) Influenza (Vaksin yang dimatikan, dilemahkan) Covid-19 (Vaksin genetik mRNA, viral vektor, dimatikan, sub-unit protein)		

This certificate is awarded to

Ms

Nurfatihah Azlyna Binti Ahmad Suhaimi

for an **ORAL PRESENTATION** titled

In vitro uptake and activation of human dendritic cells by liposomes derived from total lipid of Mycobacterium smegmatis

THE 6TH INTERNATIONAL CONFERENCE ON

MOLECULAR DIAGNOSTICS & BIOMARKER DISCOVERY

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11th – 13th October 2022
On-line (WEBEX Events)

Organised by
Institute for Research in Molecular Medicine (INFORMM)

Co-organised by
Ministry of Higher Education (MoHE) Malaysia

Dr Tye Gee Jun
Conference Chair

Assoc. Prof. Dr Aziah Ismail
Director of INFORMM