EFFECTS OF TRANSPORT INHIBITORS ON THE CELLULAR UPTAKE OF LIPOSOMES FROM *Mycobacterium Smegmatis* BY HUMAN MONOCYTES DENDRITIC CELLS

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UNIVERSITI SAINS MALAYSIA

2024

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by

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

AUGUST 2024

ACKNOWLEDGEMENT

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

I would like to express my gratitude to Allah swt for giving us opportunity and helping me endlessly in finishing this thesis entitled "Effects of transport inhibitors on the cellular uptake of liposomes from *mycobacterium smegmatis* by human monocytes dendritic cells." A special gratitude to my supervisor, Dr Ramlah Kadir, for giving me the opportunity to conduct this research project under Fundamental Research Grant Scheme (FRGS) (FRGS/1/2024/SKK10/USM/02/10) and for her guidance throughout this research. In addition, I would like to thank Prof. Madya Dr Rapeah Suppian and Dr Rohimah Mohamud, my co-supervisors, for always being available whenever I need assistance and guidance. I would like to sincerely thank the staff of the Department Immunology, especially Mr. Jamaruddin Mat Asan, Mr. Azlan Mat Nasir and Madam Nik Maziah Hussin for their assistance in the acquisition of flow cytometry. Furthermore, my utmost gratitude also goes to my research teammates Dr Lidawani, Fatmawati and Aina for always being there for me and supporting each other. Thank you for the friendship. Last but most importantly, my utmost gratitude I dedicated to my late *Abah* Nordin bin Mohamed, my *Ma* Noor Hayati binti Saleh, my husband Mohamad Yarid bin Mohd Yusoff and also my siblings for their relentless support, constant prayers, sacrifices and always encouraging me to be passionate in whatever I am doing. To my *Ma* and my late *Abah* I dedicated this achievement for both of you. I want you to know that you are the best gift I've ever received.

Nor Asyikin binti Nordin

P-UM0007/22(R)

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LIST OF SYMBOLS

℃	Degree Celsius
٪	Percent
γ	Gamma
<	Less than
>	More than
ml	Millilitre
µl	Microlitre
µm	Micrometre
nm	Nanometre
ng	Nanogram

LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
AGP	Arabinogalactan-peptidoglycan
BCG	Bacillus Calmette-Guérin
BMDCs	Bone-marrow dendritic cells
cDCs	Conventional DCs
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocytes
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DSPC	Distearoylphosphatidylcholine
dsRNA	Double-stranded RNA
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FESEM	Field emission scanning electron microscope
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA-DR	Human-leukocyte antigen D related
HPV	Human papillomavirus
IFN-γ	Interferon-gamma
IL-4	Interleukin-4
IL-12	Interleukin-12
IL-12 (p70)	Interleukin-12 (p70)

KPP	Klinik Pakar Perubatan
LPS	Lipopolysaccharides
MFI	Median Fluorescence Intensity
mRNA	Messenger ribonucleic acid
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MLVs	Multilamellar vesicles
moDCs	Monocytes derived–dendritic cells
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
NK	Natural killer
PAMPs	Pathogen-associated molecular patterns
PBMC	Human peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid DCs
PKA	Protein kinase A
PPSP	Pusat Pengajian Sains Perubatan
PRRs	Pattern recognition receptor
RPMI	Rosewell Park Memorial Institute
SEM	Scanning electron microscope
SLN	Solid lipid nanoparticles
SUVs	Small unilamellar vesicles
TB	Tuberculosis
TCR	T cell receptor

TEM	Transmission electron microscope
Th1	Type 1 T helper
Th2	Type 2 T helper
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
USM	Universiti Sains Malaysia
VADS	Vaccine adjuvant delivery system
VLP	Virus-like particle

LIST OF APPENDICES

KESAN PERENCATAN PENGANGKUTAN TERHADAP PENGAMBILAN SELULAR LIPOSOM DARIPADA *Mycobacterium smegmatis* OLEH SEL DENDRITIK MONOSIT MANUSIA

ABSTRAK

Nanoteknologi seperti liposom dijangka memainkan peranan penting dalam bidang perubatan nano yang berkembang pesat. Liposom ialah sejenis nanopartikel berasaskan lipid yang dibentuk oleh pemasangan sendiri molekul fosfolipid dalam medium berair. Pada masa kini, ia digunakan secara meluas sebagai adjuvant atau pengangkutan untuk menyampaikan pelbagai jenis agen terapeutik. Liposom adalah biokompatibel, terbiodegradasi dan pengeluaran kos yang lebih rendah, jadi disebabkan oleh faktor ini, banyak perhatian ditumpukan kepada mereka sebagai pembawa yang berpotensi untuk penghantaran yang disasarkan dalam penyelidikan bioperubatan untuk kesan adjuvantnya pada sel dendritik (DCs). DCs dikenali sebagai sel pembentang antigen profesional (APCs), yang boleh menyerap, memproses dan mempersembahkan antigen. DCs berada di pusat sistem imun dan mampu berinteraksi dengan kedua-dua sel B dan sel T, dengan itu memanipulasi humoral dan tindak balas imun selular. Ia menyediakan hubungan penting antara imuniti semulajadi dan adaptif imuniti. Oleh kerana fungsi pengaktifan imun yang kuat dan sifat adjuvant semula jadi, ia menjadikan mereka sasaran yang berharga untuk penghantaran antigen. Berdasarkan penulisan, beberapa kajian menyiasat tentang potensi liposom yang diperoleh daripada *Mycobacterium smegmatis* (*M. smegmatis*) (liposomes-Msmeg) sebagai vaksin berasaskan lipid telah dilaporkan, namun mekanisma pengambilan liposom-Msmeg oleh DCs monosit manusia daripada sel mononuklear darah peripheral (PBMC) dan permulaan tindak balas

imun adaptif belum ditemui sepenuhnya. Oleh itu, kami berhasrat untuk menyiasat mekanisma pengambilan selular liposom semulajadi *M. smegmatis* sebagai vaksin berasaskan lipid oleh DCs. Untuk menilai mekanisma pengambilan selular liposom-Msmeg, perencat khusus seperti chlorpromazine hydrochloride, filipin III dan cytochalasin B yang menghalang laluan tertentu akan digunakan. Dalam kajian ini, PBMC daripada individu yang sihat akan dikumpulkan untuk mengkaji populasi monosit mengikut aliran sitometri, manakala mekanisma pengambilan liposom-Msmeg akan dilihat dengan mengimbas mikroskop elektron (FESEM) dan mikroskop konfokal. Supernatan akan dikumpulkan untuk mengukur tahap sitokin oleh ELISA. Oleh itu, dalam kajian yang dicadangkan ini, kami akan mengisi jurang dengan menyiasat mekanisma pengambilan liposom-Msmeg sebagai vaksin berasaskan lipid yang kuat oleh DCs daripada PBMC dalam individu yang sihat.

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ABSTRACT

Nanotechnology such as liposomes is expected to play an important role in the rapidly growing field of nanomedicine. Liposomes are a type of lipid-based nanoparticles formed by the self-assembly of phospholipid molecules in an aqueous medium. Nowadays, it is widely used as an adjuvant or even a vehicle to deliver various therapeutic agents. Liposomes are biocompatible, biodegradable and have lower production costs, so due to these factors, much attention is devoted to them as potential carriers for targeted delivery in biomedical research for their adjuvant effects on dendritic cells (DCs). DCs are known as professional antigen-presenting cells (APCs), which can absorb, process and present antigens. DCs are at the centre of the immune system and capable of interacting with both B cells and T cells, thereby manipulating the humoral and cellular immune responses. It provides an essential link between innate and adaptive immunity. Due to strong immune activation function and natural adjuvant properties, it makes them a valuable target for antigen delivery. Based on literature, few studies investigating on the potential of liposomes derived from *Mycobacterium smegmatis* (*M. smegmatis*) (liposomes-Msmeg) as a lipid-based vaccine have been reported, however the uptake mechanism of liposomes-Msmeg by human monocytes DCs from human peripheral blood mononuclear cells (PBMC) and initiation of adaptive immune response have not been fully discovered. Thus, we aimed to investigate the cellular uptake mechanism of natural liposomes-Msmeg as a lipid-based vaccine by DCs. To assess cellular

uptake mechanisms of liposomes-Msmeg, specific inhibitors such as chlorpromazine hydrochloride, filipin III and cytochalasin B which inhibit the specific pathway will be used. In this study, PBMC of healthy individuals was collected and sorted to study the monocyte populations by flow cytometry, while uptake mechanisms of liposomes-Msmeg will be viewed by field emission scanning electron microscope (FESEM) and confocal microscopy. Supernatant will be collected to measure the cytokine level by ELISA. Therefore, in this proposed study, we are going to fill up the gaps by investigating the uptake mechanisms of liposomes-Msmeg as a potent lipid-based vaccine by DCs from PBMC in healthy individuals.

CHAPTER 1

INTRODUCTION

1.1 Study background

Liposomes are nanoparticles that have a single sphere-shaped vesicle with a range size from 0.025 µm to large 2.5 µm vesicles (Akbarzadeh et al., 2013a). Liposomes are usually made from synthetic lipids, semi-synthetic lipids, or natural lipids from microorganisms, including bacteria, depending on their desired characteristics and application (Dymek & Sikora, 2022; Nagalingam, 2017). Liposomes may develop by one or bilayer membrane which the number of bilayers and their size affects the amount of uptake macromolecules such as proteins, deoxyribonucleic acid (DNA) and imaging agents (Guimarães et al., 2021; Mosquera et al., 2018; Sharma et al., 2018). Liposomes are effective drug delivery vehicles for various diseases, especially in infectious diseases and cancer (Nisini et al., 2018; X. Pan et al., 2021; T. S. Patil & Deshpande, 2020; Rommasi & Esfandiari, 2021; Saraf et al., 2020; A. P. Singh et al., 2019).

In this study, we successfully synthesised liposomes from bacterial lipids of *Mycobacterium smegmatis (M. smegmatis)* in the range of 20-80 nm, which are classified as small unilamellar vesicles (SUV). The study found that SUVs are a good choice for cellular uptake by human monocytes derived–dendritic cells (moDCs) (Luwi et al., 2020). *M. smegmatis* is commonly used as a model organism in cell culture labs to study other mycobacteria species such as *Mycobacterium tuberculosis* (*M. tuberculosis*), *Mycobacterium bovis* and *Mycobacterium fortuitum* due to non-pathogenic, rapid growth, genetic similarity, genetic compatibility, cell wall composition and drug targeting (Ranjitha et al., 2020). A previous study found that liposomes from *M. smegmatis* were effective in delivering the anticancer drug

doxorubicin to human breast cancer cells (Apolinario et al., 2021; A. Li et al., 2021; Makowski et al., 2019). The liposomes also reduced the side effects of doxorubicin (Ansari et al., 2017). Another study by Kaur et al. (2018) and Minakshi et al. (2019) found that liposomes from *M. smegmatis* were effective in delivering a vaccine against tuberculosis (TB) to mice (Kaur et al., 2022; Minakshi et al., 2019). Liposomes also proved that they can improve the immunogenicity of vaccines and protect mice from TB infection (Kaur et al., 2022; Minakshi et al., 2019). These studies demonstrate that liposomes from *M. smegmatis* are a promising new technology for drug and vaccine delivery.

moDCs are a type of immune cell derived from monocytes that play an essential role in both innate and adaptive immunity (Puck et al., 2015). moDCs are phagocytic cells, which engulf particles ranging in size from 20 nm to 200 nm in diameter (Matveeva et al., 2022; Silva et al., 2017). moDCs are important targets for liposome-based delivery systems in the manufacture of vaccines because they facilitate both inducing and controlling the immune response by starting and modifying the body's defence processes (Bell & Kutzler, 2022; Stolk et al., 2020). moDCs induce immune activation by taking up antigens by phagocytosis or endocytosis and processing the antigens efficiently. The antigenic peptides are displayed on the moDCs surface by using major histocompatibility complexes (MHC), either MHC I or II, and recognised by T cells passing through the lymph nodes. The recognition of antigenic peptides by T cells allows the activation of the immune response (Ho et al., 2018). At the same time, moDCs control the immune response by increasing the T cell anergy, producing immunosuppressive cytokines such as TGF-β and IL-10, and inducing tolerance through regulatory T cells (Tregs). By maintaining self-tolerance and avoiding detrimental overreactions, these systems

guarantee that the immune system can react to infections in an efficient manner. moDCs are also important in recognising vaccine-derived antigens, where they help the immune system develop specific and long-lasting memories against pathogens, which is essential for vaccine efficacy (Mansouri et al., 2021).

moDCs take up liposomes through three main mechanisms: receptor-mediated endocytosis, phagocytosis and macropinocytosis (Warrier et al., 2019). This allows liposomes to be efficiently taken up and internalised by moDCs, which supports their function in antigen presentation and further promotes the production of an immune response (Affandi et al., 2020). Liposomes on the cell surface bind to specific receptors on moDCs (Affandi et al., 2020). This binding triggers the formation of clathrin-coated pits or caveolae (indentations in the cell membrane). These indentations then pinch off, enclosing the liposome and bringing it inside the moDC. The second mechanism is called phagocytosis, which is used for engulfing large particles, such as whole bacteria (Lee et al., 2020). It's less likely to be the primary mechanism for liposome uptake unless the liposomes are very large. Third, macropinocytosis, which refers to engulfing fluids and solutes in bulk through large vesicles called macropinosomes (Freeman & Grinstein, 2018). Liposomes might be internalised along with surrounding fluid through this mechanism.

Investigating cellular uptake of liposomes involves using inhibitors such as cytochalasin B, chlorpromazine and filipin III are often used in research studies to investigate the cellular uptake of various molecules and particles (de Almeida et al., 2021; De Ruiter et al., 2019; Nagy et al., 2022; Nelemans & Gurevich, 2020; Saw et al., 2018). Each of these compounds specifically targets and inhibits a distinct pathway involved in cellular uptake (Nagy et al., 2022). Previous studies have shown that cytochalasin B inhibits endocytosis and phagocytosis by disrupting the actin

cytoskeleton (Kokhanyuk et al., 2021; Lai & Wong, 2020). The actin cytoskeleton is crucial for many cellular processes, including the formation of vesicles used for endocytosis and phagocytosis (Sˇamaj et al., 2004). Disruption of the actin cytoskeleton prevents cells from effectively internalising molecules and particles from the extracellular environment (Kokhanyuk et al., 2021; Lai & Wong, 2020).

Chlorpromazine inhibits clathrin-mediated endocytosis by interacting with clathrin, a protein involved in forming clathrin-coated pits, which are the entry sites for clathrin-mediated endocytosis (F. Chen et al., 2018). Interference with clathrin prevents the formation of clathrin-coated pits, thereby blocking the internalisation of molecules and particles via clathrin-mediated endocytosis. Filipin III inhibits caveolae-mediated endocytosis by disrupting caveolae (Alkafaas et al., 2023), which are small invaginations of the plasma membrane that are involved in caveolae-mediated endocytosis (Alkafaas et al., 2023). Disruption of caveolae prevents cells from effectively internalising molecules and particles via caveolae-mediated endocytosis. The use of these inhibitors in research provides valuable insights into the mechanisms and regulation of cellular uptake. By selectively blocking specific pathways, researchers can determine the relative contributions of each pathway to the uptake of different molecules and particles (Dos Santos et al., 2011). Additionally, these inhibitors can be used to study the effects of cellular uptake on various cellular processes, such as signalling and gene expression (Zhao et al., 2011).

In order to investigate the potential of liposomes from *M. smegmatis* as a lipid-based vaccine in humans, we will focus our investigation on the effects of transport inhibitors on the cellular uptake of these liposomes from *M. smegmatis* by human moDCs. Therefore, this study would help us to establish a greater understanding in the activation of the immune system via the cellular interaction of such liposomes to

moDCs, which can further accelerate the development of liposomes from *M. smegmatis* as therapeutic medicine.

1.2 Problem statement

Vaccines are biological substances that provide actively acquired immunity to certain infectious diseases. It typically contains an agent that resembles disease-causing microorganisms and it is made from weak or dead forms of microbes, toxins or surface proteins. The development of vaccines involves multiple platforms that have their unique approach in stimulating the immune system to recognise and fight pathogens. The main platforms used in vaccine development are as follows live attenuated vaccines, inactivated (Killed) vaccines, toxoid vaccines, messenger ribonucleic acid (mRNA) vaccines, viral vector vaccines, DNA vaccines, protein subunit vaccines and virus-like particle (VLP) vaccines. The most advanced platform now is the use of mRNA in the production of COVID-19 vaccine where the development has significantly accelerated during pandemic to prevent the COVID-19 infection from spreading quickly which subsequently reduced the mortality rate at that time. There are over 25 licensed vaccines are now available in the market to prevent various infectious diseases caused by viral infections such as hepatitis A, human papillomavirus (HPV), COVID-19, measles, mumps, rubella, polio, etc., and bacterial infections such as pneumococcal disease, meningococcal disease, typhoid, cholera, tetanus, diphtheria, etc. However, vaccine research and development has expanded to include non-infectious diseases such as cancer, allergies and autoimmune diseases. However, the platform used in the production of these vaccines does not specifically utilise adjuvants or delivery systems as part of the vaccine formulation and are not specifically designed to target dendritic cells (DCs). Liposomes are a powerful tool in

vaccine development due to their versatility (Nisini et al., 2018). They can encapsulate vaccine antigens, protecting them from degradation in the body (Nisini et al., 2018). This can be especially important for antigens that are fragile or prone to breakdown (Filipczak et al., 2020). Additionally, liposomes can co-deliver adjuvants, substances that strengthen the immune response, alongside the antigens (Gu et al., 2020). Co-delivering adjuvants with antigens significantly enhances the vaccine's potency (Gu et al., 2020). This controlled release of antigens allows for a sustained immune response over time (Lima & Rodrigues Junior, 1999). Liposomes can be modified to target specific cells in the body (Yan et al., 2020). This can help to improve the delivery of antigens to the cells that are most important for generating immunity (Yan et al., 2020). Liposomes are made from natural materials, making them a safe and biocompatible option for vaccine delivery (Karunakaran et al., 2023). With these advantages, liposomes hold great promise for the creation of next-generation vaccines. Development of vaccines by targeting DCs is one of the promising strategies to enhance their activation and antigen presentation capabilities, leading to a robust immune response. DCs are specialised APCs that take up the antigens or pathogens, and internalise the antigens by a variety of processes, including receptor-mediated endocytosis, phagocytosis and macropinocytosis. In addition, the use of adjuvants or delivery systems in vaccine formulations can further promote the uptake of antigens by DCs and further increase vaccine efficacy. Therefore, this study focused on investigation of the effect of transport inhibitors on the cellular uptake of liposomes from *M. smegmatis* by human moDCs. By investigating the inhibitory effect on the uptake of liposomes from *M. smegmatis* by human moDCs, it is possible to gain insight into the mechanism of uptake by moDCs that can lead to the activation of an immune response.

1.3 Research objectives

1.3.1 General objective

To investigate the effects of transport inhibitors on the cellular uptake of liposomes from *M. smegmatis* by human monocytes dendritic cells.

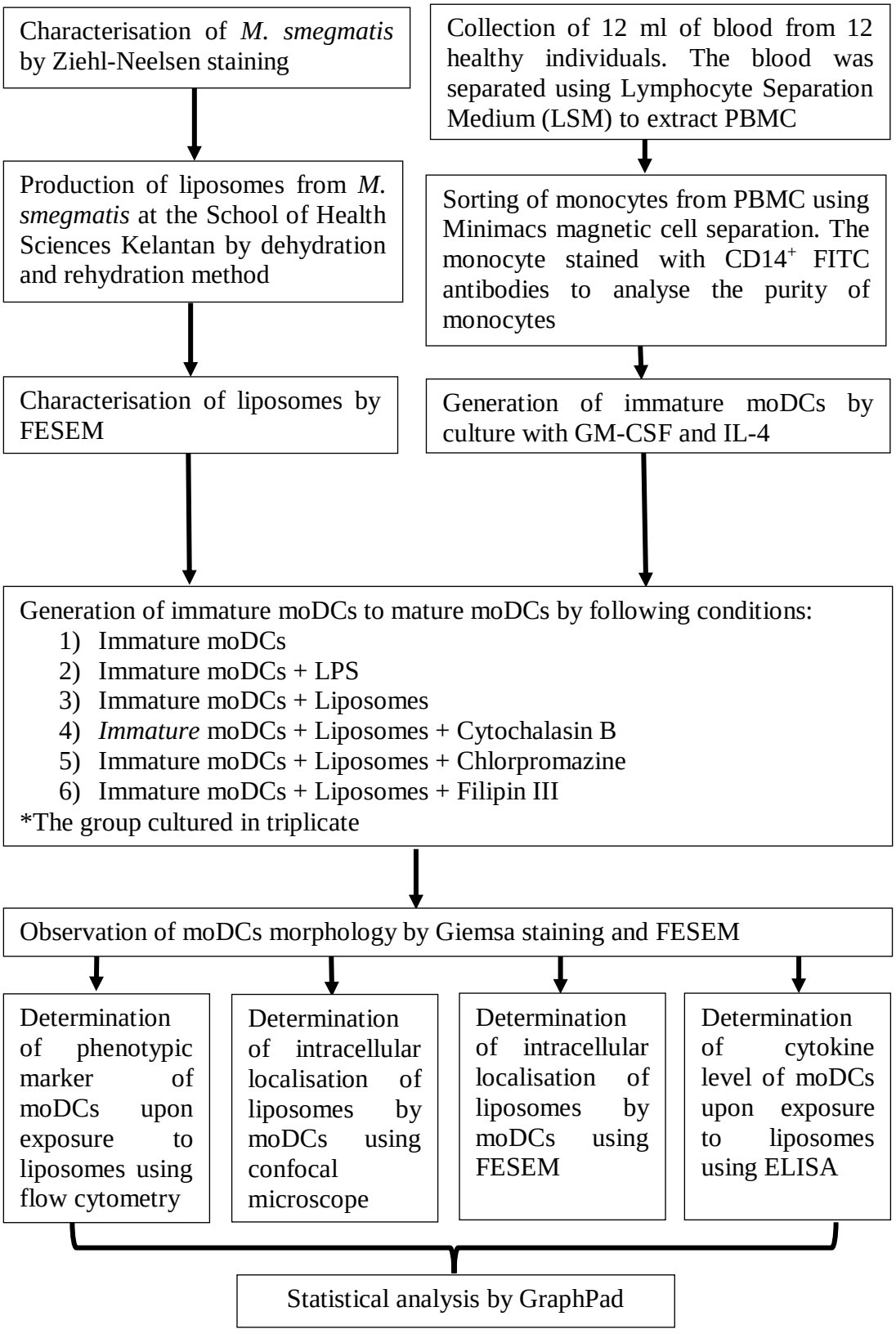
1.3.2 Specific objective

1. To produce and characterise liposomes from the total lipid of *M. smegmatis*.
2. To determine the intracellular localisation of liposomes from *M. smegmatis* by moDCs upon exposure to liposomes and inhibitor; cytochalasin B, chlorpromazine and filipin III using field emission scanning electron microscope (FESEM) and confocal microscope.
3. To determine the phenotypic markers of moDCs; HLA-DR, CD11c, CD80 and CD86 upon exposure to liposomes and inhibitors; cytochalasin B, chlorpromazine and filipin III using flow cytometry.
4. To determine secretion level of cytokines; IFN-y, IL-4 and IL-12 (p70) upon exposure to liposomes and inhibitors; cytochalasin B, chlorpromazine and filipin III by ELISA.

1.3.3 Research hypothesis

The uptake of liposomes from *M. smegmatis* by human monocytes dendritic cells will be inhibited by transport inhibitors and they will affect the expression of phenotypic markers and the secretion levels of cytokines.

1.4 Flow chart of the study



CHAPTER 2

LITERATURE REVIEW

2.1 Immune response

The immune system is a complex system that protects the body from foreign invaders, such as bacteria, viruses, parasites and toxins (Jafari et al., 2019). The immune system employs various responses tailored to fight specific types of pathogens (Rankin & Artis, 2018). They respond to diverse infections and environmental stimuli through distinct pathways and processes that are categorised as type 1, type 2 and type 3 immunity. These 3 types of immunity are characterised by different immune cells, effector functions and cytokines. Type 1 immunity is mainly involved in defence against intracellular pathogens and characterised by the production of cytokines, such as interferon-gamma (IFN-γ), that help to kill the invaders (Rankin & Artis, 2018; X. Zhu & Zhu, 2020). Particularly, type 2 immunity is prominent in fighting helminth infections by promoting the production of antibodies by B cells and activating eosinophils to expel parasites, but are not effective against bacteria (Coakley & Harris, 2020). This response is characterised by the production of interleukin-4 (IL-4), which helps to activate the immune system and promote the expulsion of the worms (Rankin & Artis, 2018). The other type is type 3 immunity, which contributes to defending against extracellular bacteria and fungi that are characterised by the production of IL-17 and IL-22. This immune response is also associated with autoimmune and inflammatory diseases. While some antibodies might also be involved, they play a less prominent role compared to the direct cellular attack (Agerholm & Bekiaris, 2021)**.** Immune cells and other cells produce a variety of soluble molecules, including cytokines, hormones and neuropeptides (Shouman & Benarroch, 2021). These molecules allow immune cells to sense, process and relay

signals from the body beyond their role in fighting pathogens and tumours (Rankin & Artis, 2018).

2.1.1 Innate immune response

The innate immune system is the body's first line of defence against infection (Diamond & Kanneganti, 2022). It is a rapid and nonspecific response that does not require prior exposure to the pathogen (Aristizábal & González, 2013). It is made up of both physical and cellular components (Aristizábal & González, 2013). The physical components include the skin and mucous membranes, which act as a barrier to prevent pathogens from entering the body (Aristizábal & González, 2013). The cellular components include cells such as phagocytes and natural killer (NK) cells, which can recognise and destroy pathogens (Akdis et al., 2020).

The innate immune system utilises three primary methods to identify and respond to invading pathogens (Figure 2.1): pattern recognition receptors (PRRs), damage-associated molecular patterns (DAMPs), and innate immune receptors (D. Li & Wu, 2021). PRRs are a large family of proteins that can recognise pathogen-associated molecular patterns (PAMPs) and DAMPs. PRRs recognise PAMPs, which are signature molecules found on microbes but not typically on human cells (D. Li & Wu, 2021), while DAMPs are molecules released not just by pathogens but also by stressed or damaged host cells (Patel, 2018). PAMPs and DAMPs are able to alert the immune system to potential danger, even from within the body itself (Patel, 2018).

The innate immune system recognises common molecular patterns associated with pathogens PAMPs and triggers a number of responses, including inflammation and phagocytosis (Mukherjee et al., 2023). Inflammation is a localised response that brings white blood cells and other immune cells to the site of infection (Inggarsih &

Hidayat, 2023). PRRs can initiate it to identify microbes or damaged tissue (Roh & Sohn, 2018). PRRs trigger a cascade of events that leads to the activation of immune cells and initiating an immune response (Roh & Sohn, 2018).

Phagocytosis is the process of engulfing and destroying foreign particles by cells called phagocytes (Mukherjee et al., 2023). The cells involved in this process are antigen-presenting cells (APCs) including DCs, macrophages and B cells. All APCs are phagocytes; not all phagocytes (like neutrophils) are professional APCs specialised for antigen presentation. DCs are professional APCs compared to other cells (Schuijs et al., 2019). When a phagocyte engulfs a pathogen, it breaks down the pathogen into small peptides (Guerriero, 2019). These peptides are then presented on the surface of the phagocyte by MHC class II molecules (Guerriero, 2019). MHC class II molecules are proteins that are found on the surface of DCs (Guerriero, 2019). Macrophages and B cells can also upregulate MHC class II molecule expression under certain conditions, such as during inflammation or infection (Y. Li et al., 2019). Previous study stated that there were no significant differences between the efficiency of antigen presentation by macrophages, B cells and DCs (Hua & Hou, 2020; Rastogi et al., 2022).

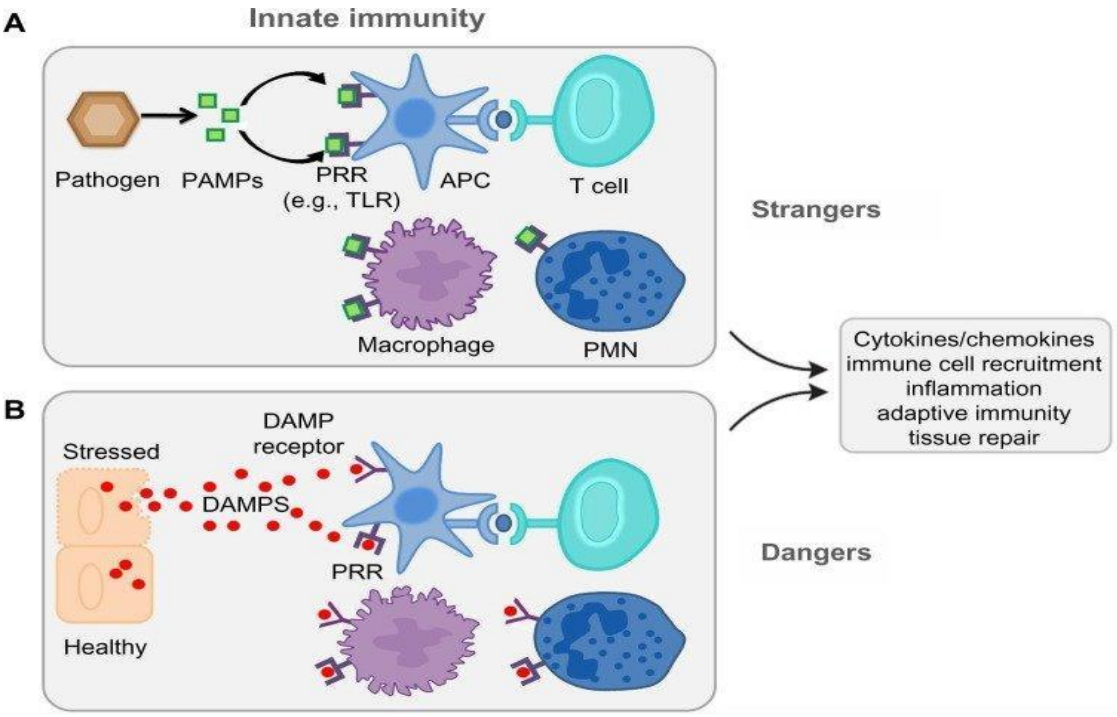


Figure 2.1 The subsystems of immune responses: Innate immunity

Models for the release of PAMPs that bind to PRRs, such as TLRs, on immune cells to stimulate an innate immune response by (A) infections of pathogens or viruses and (B) events occur when the cells are stressed or injured. (Adapted from: Vallés et al., 2014)

2.1.2 Adaptive immune response

Adaptive immune response is a second-line defence of response that can memorise and recognise the microbial and non-microbial substances, which differs from innate immunity (Sá-Nunes, 2022). The adaptive immune response is made up of cells that are specific for each pathogen, toxin, or allergen (Flayer et al., 2021). The development of this type of immunity is often slow to eliminate microorganisms as it involves several processes, including gene activation, protein synthesis and cell proliferation, in order to reach sufficient numbers to mount an effective response against the antigen (Abbas et al., 2019; Gbabe et al., 2014). There are two main mechanisms in the adaptive immune responses, which are humoral and cell-mediated immunity (Kurup et al., 2019).

2.1.2(a) Humoral immunity

Humoral immunity is a type of adaptive immunity that uses antibodies to defend the body against pathogens like bacteria, viruses, and parasites (Figure 2.2) (Abbas, 2014). B cells are the key players in humoral immunity and produce large quantities of specific antibodies upon encountering an antigen (Inoue et al., 2018). When a B cell encounters an antigen, it activates and differentiates into either plasma cells or memory B cells (Abbas, 2014). Plasma cells are responsible for producing large amounts of antibodies for a specific antigen (Abbas, 2014). These antibodies are released into the bloodstream and lymph fluid, where they can bind to pathogens and neutralise them (McComb et al., 2019). Then, memory B cells are known as the body's "immune memory". They can remember the antigen that activated them. They allow the body to fight off infections more quickly and efficiently after being exposed to them.

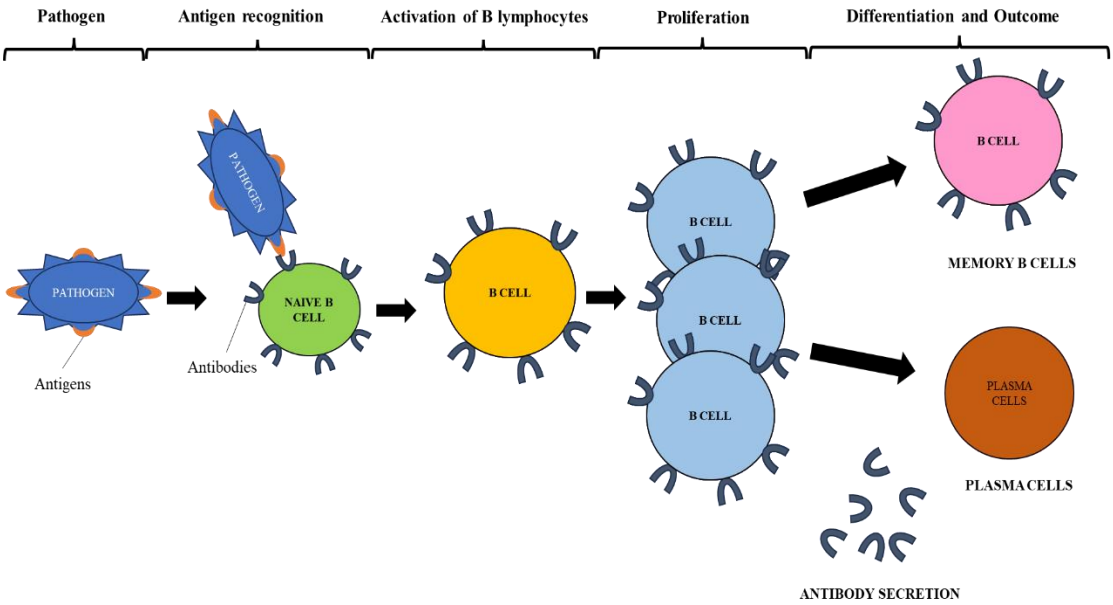


Figure 2.2 The subsystems of immune responses: Humoral immunity and antibody production

2.1.2(b) Cell-mediated immunity

Cell-mediated immunity, a type of adaptive immune response, is mainly mediated by helper T cells, specifically $CD4^+$ T cells. These cells can be further classified into subtypes based on the cytokines they secrete and the immune response they promote (Ahlborga, 2023; Jansen et al., 2019).

Based on figure 2.3, Type 1 T helper (Th1) cells are primarily activated when their T cell receptor (TCR) recognises a foreign antigen fragment bound to an MHC class II molecule on an antigen-presenting cell (APC) (Fasano et al., 2022). They primarily combat intracellular pathogens by secreting cytokines like IFN-γ, which activate macrophages and cytotoxic T cells (Valle-Noguera et al., 2021). Type 2 T helper (Th2) cells, on the other hand, are crucial for fighting extracellular pathogens and allergens (X. Zhu & Zhu, 2020). They promote antibody production by B cells through IL-4, but they are not directly involved in MHC molecule recognition (Rochman et al., 2018).

The type of APCs and antigen encountered determines the specific cytokines released by Th cells, which then activate other immune cells, such as B cells and $CD8^+$ cytotoxic T cells (Hua & Hou, 2020; Ranga et al., 2020). B cells are activated by Th2 cells. Th2 cells secrete IL-4 cytokine, which stimulates B cells to mature into plasma cells, specialised for producing large amounts of antibodies (Rochman et al., 2018). Antibodies bind to the pathogens and neutralise them (Marshall et al., 2018).

The activation of $CD8^+$ cytotoxic T cells can be initiated when a $CD8^+$ cytotoxic T cell binds to an MHC I-epitope complex on an infected cell (Ranga et al., 2020). After activation, they produce granzymes and perforins. Granzymes are a family of proteases that can break down proteins in a cell, and perforins create holes in the plasma membrane of an infected cell (Barry & Bleackley, 2002). This allows the

granzymes to enter the cell, leading to cell death (Ritter et al., 2022). The process of $CD8^+$ cytotoxic T cell killing is called cytolysis, which is essential for clearing infections from the body (Ritter et al., 2022).

The induction of strong cytotoxic T cells and memory responses by Th1 depends on $CD8^+$ cytotoxic T cells. They produce cytokines, such as IFN-γ, that activate macrophages and cytotoxic T cells (Anderson & Simon, 2020). Th1 cells also secrete tumour necrosis factor alpha (TNF-α), which can promote inflammation and cell death (Guo et al., 2019). However, cytokines play a critical role in regulating the immune response and maintaining a healthy immune system (Upadhyay et al., 2018).

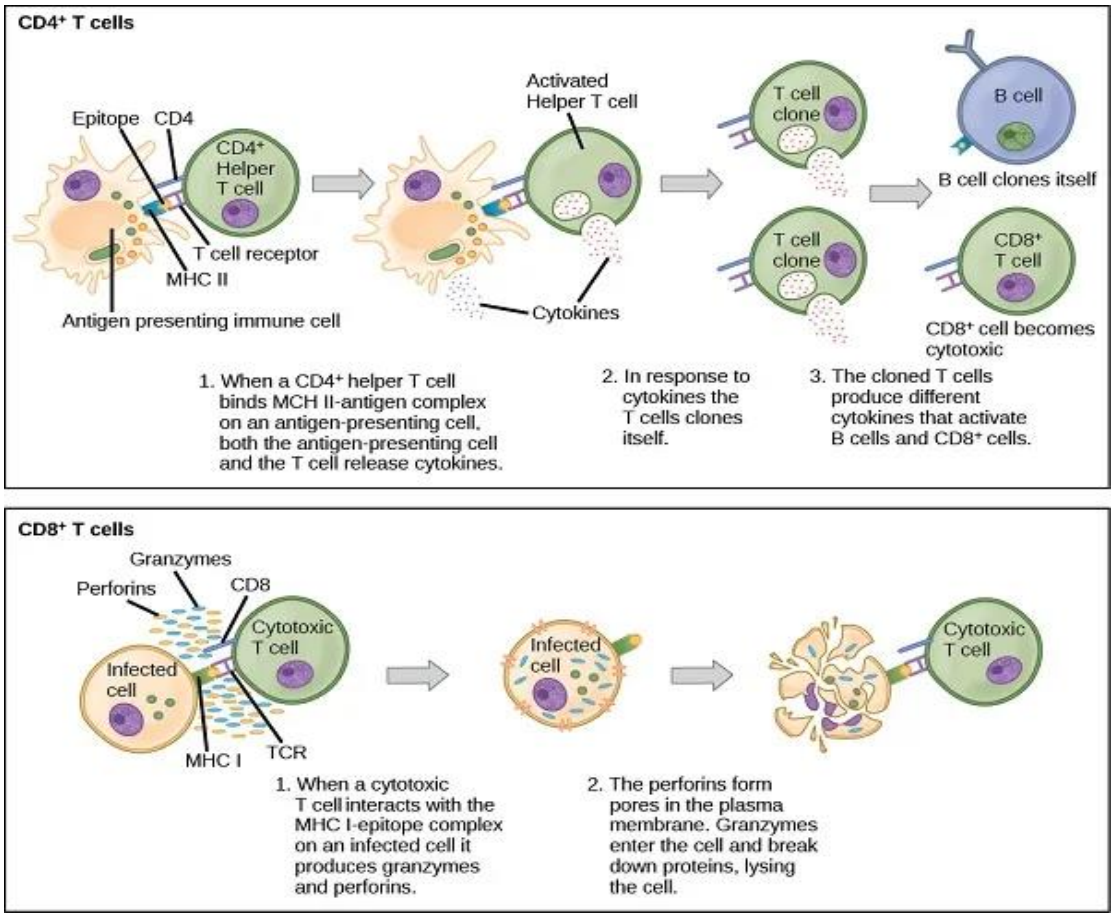


Figure 2.3 Naive T cells expressed on one of two different molecules, $CD4^+$ and $CD8^+$, on APCs surface

Naive $CD4^+$ T cells interact with MHC II molecules on APCs to be activated. In turn, the cloned activated helper T cells activate B cells and $CD8^+$ T cells, which become

cytotoxic T cells. Cytotoxic T cells kill infected cells. Adapted from (Molnar & Gair, 2013)

2.2 Antigen-presenting cells

Antigen-presenting cells are a bridge between innate and adaptive immune systems (Mateos-Maroto et al., 2023). APCs consist of DCs, macrophages, and B cells (Kordon et al., 2018). They are found throughout the body, including in the skin, lungs and intestines (Jakubzick et al., 2017). APCs able to capture, process, and present antigens to T cells (Schuijs et al., 2019). This process is essential for the initiation of the adaptive immune response. However, there are some key differences between these three types of APCs (Iadecola et al., 2020). DCs are known as professional APCs that play a sentinel role in the immune system (Tatiya-Aphiradee et al., 2018). They are the most dominant due to their advanced ability in priming naive T cells which stimulates the primary immune response (Lin & Loré, 2017; Shedlock & Weiner, 2000). DCs are able to capture a wide variety of antigens, including bacteria, viruses, and tumour cells. DCs then process these antigens and present them to T cells to trigger immune response (Y. Wang et al., 2020).

Macrophages are phagocytic cells, meaning that they are able to engulf and destroy foreign particles, including bacteria, viruses, and dead cells (Akata & van Eeden, 2020). Macrophages can also process and present antigens to T cells (Akata & van Eeden, 2020). However, macrophages are less efficient at antigen presentation than DCs (Muntjewerff et al., 2020).

B cells are primarily known for their role in producing antibodies, but they can also function as APCs (Rydzewska et al., 2018). B cells are able to capture, process and present these antigens to T cells in a form that can be recognised and triggered

immune response (Marshall et al., 2018). However, B cells are less efficient at antigen presentation than DCs and macrophages (Cruse et al., 2004).

Overall, DCs, macrophages, and B cells are important APCs that play a vital role in the immune system (Cruse et al., 2004). However, they have different functions and roles in antigen presentation and other aspects of the immune system. DCs are the most important APCs for initiating the adaptive immune response to infection. Macrophages are more important for phagocytosing and destroying foreign particles. B cells are more important for producing antibodies against specific antigens (Cruse et al., 2004).

2.3 Dendritic cells

Dendritic cells, known as the sentinels of the immune system due to their crucial role in antigen presentation, are also considered promising targets for vaccine-based immunotherapy (Mateos-Maroto et al., 2023; Qian & Cao, 2018). DCs are classified into three main categories: monocytes derived–dendritic cells (moDCs), plasmacytoid DCs (pDCs), and conventional DCs (cDCs) (J. Liu et al., 2020). These subsets have distinct functions, contributing to the diverse immune response mediated by DCs (Leylek et al., 2020). While these subsets have unique phenotypic and functional features, the transcriptional programs that determine their identities remain elusive. DCs play a pivotal role in activating the immune response through a division of labour among functionally specialised subsets (Leylek et al., 2020).

DCs excel at presenting antigens on both MHC class I and class II molecules (Kotsias et al., 2019). MHC molecules are proteins found on the surface of most cells that act as identification tags, helping the immune system differentiate between healthy body cells and infected cells or foreign invaders (Huitema et al., 2021). DCs

are particularly adept at presenting antigen fragments on both MHC class I and class II molecules, allowing them to interact with various T cells (Embgenbroich & Burgdorf, 2018; Jurewicz & Stern, 2019).

DCs play a dominant role in the induction of immunity by presenting peptides on MHC class I molecules through direct presentation or "cross-presentation." They express significant levels of MHC class I products, which is essential for stimulating antigen-reactive $CD8^+$ cytotoxic T cells (Embgenbroich & Burgdorf, 2018). MHC class II molecules are presented after antigen uptake and processing within the endocytic pathway. This process begins during the selection stage of T cell development in the thymus and continues in the periphery (Jurewicz & Stern, 2019). $CD4^+$ T helper cells, on the other hand, are activated by processed antigens presented on MHC class II molecules by other APCs like B cells in the periphery. This is essential for their survival and function (Jurewicz & Stern, 2019).

Surface markers of DCs are unique molecules that are expressed on the surface of these immune cells (Balan et al., 2019). These markers play a crucial role in various aspects of DCs function, including antigen capture, processing, and presentation, as well as cell adhesion, migration, and interaction with other immune cells (Kotsias et al., 2019). Some examples of human DCs surface markers are HLA-DR, CD11c, CD80, and CD86. CD80 and CD86 are accurately classified as co-stimulatory markers, while HLA-DR is primarily an antigen presentation marker, and CD11c is a general marker for certain immune cells. These are just a few examples, and there are many other human surface markers with diverse functions. By studying and utilising these markers, researchers can advance our knowledge of the immune system and develop novel therapeutic approaches for various diseases.

2.3.1 Monocyte-derived dendritic cells

Monocyte-derived dendritic cells exist in two main states: immature and mature (Balan et al., 2019). Immature moDCs are inefficient at activating T cells due to low levels of co-stimulatory molecules and immunostimulatory cytokines on their surface. This can lead to T cell anergy or deletion, which is important for immune health (Schmidt et al., 2012; Y. Xing & Hogquist, 2012). Thus, immature moDCs are not suitable for use in immunotherapy (Yi, 2021). Immature moDCs play a vital role in immune surveillance. They are adept at antigen uptake while patrolling tissues (Arasa et al., 2021). However, their inability to activate T cells is crucial for maintaining immune tolerance (Cools et al., 2008). This ensures that T cell responses only occur in the presence of danger signals, preventing reactions to harmless substances and potential autoimmunity (Cools et al., 2008).

This regulatory function ensures that T cell activation occurs only in the presence of appropriate stimuli, safeguarding against autoimmunity, and hyperactivation (Cools et al., 2008)**.** The transition of immature moDCs to mature moDCs is essential for efficient T cell activation, as mature moDCs possess the necessary molecular machinery and signalling pathways to effectively prime and activate T cells, initiating adaptive immune responses (Nowatzky et al., 2018)**.**

In contrast to immature moDCs, mature moDCs are fully developed and highly effective at activating T cells (X. Chen et al., 2018). They offer several advantages: they can be obtained and generated in large numbers, are highly adaptable, and can differentiate into various subsets with specialised functions (Zanna et al., 2021). Most importantly, mature moDCs efficiently present antigens to T cells, triggering a strong and effective immune response against invading pathogens (Dudek et al., 2013).

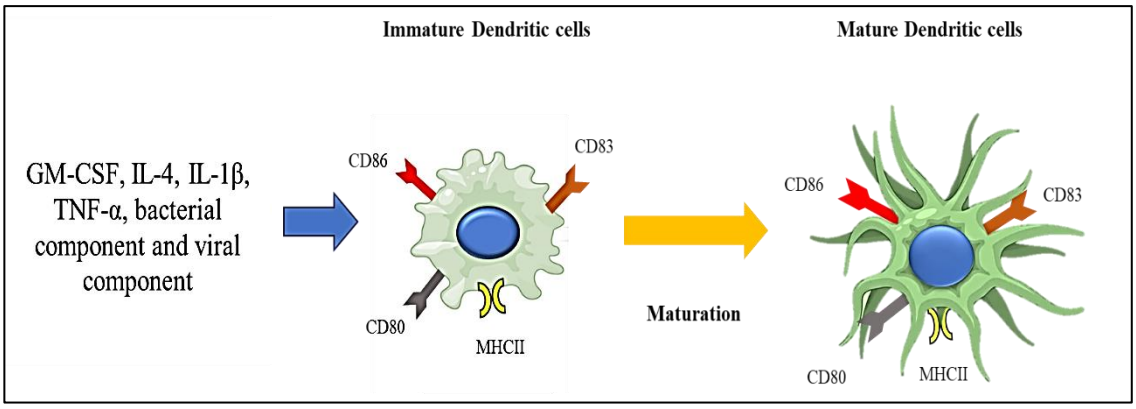


Figure 2.4 Maturation of moDCs triggered by proinflammatory cytokines, bacterial components and viral components

The maturation of moDCs is triggered by various environmental factors. These include proinflammatory cytokines e.g., granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-1β, TNF-α, bacterial components e.g., lipopolysaccharides (LPS), flagellin, peptidoglycan, CpG DNA, and viral components e.g., double-stranded RNA (dsRNA), CpG DNA, viral proteins (Ellis et al., 2005; Onomoto et al., 2021; Sehgal et al., 2020). These bacterial and viral components are recognised by PRRs on the surface of moDCs, as shown in figure 2.4. When a PRR binds to a pathogen-associated molecular pattern (PAMP), it triggers a signalling cascade that leads to moDC maturation (Schreibelt et al., 2010).

The maturation, activation, and immunostimulatory capacity of moDCs are accompanied by distinct phenotypic changes. These changes include upregulation of MHC molecules, enhanced production of inflammatory cytokines and co-stimulatory molecules (Schmidt et al., 2012). Due to their ability to generate a strong immune response, mature moDCs are actively being explored for developing vaccines and immunotherapies against infectious diseases (Cox et al., 2020).

2.3.2 Generation of moDCs

Monocytes isolated from PBMC can generate mature moDCs. Magnetic separation, a technique that enriches monocytes through positive or negative selection, can further purify the starting population (Bhattacharjee et al., 2017). Positive selection of monocytes is often preferred to avoid interference in moDC generation (Chometon et al., 2020). Protein supplementation such as penicillin-streptomycin solution to prevent microbial contamination, along with recombinant human GM-CSF, recombinant human IL-4, and complete media, are added to culture media to promote cell growth and differentiation of moDCs (Jacobs et al., 2008). Different combinations of signalling molecules, such as cytokines, growth factors, and adjuvants, can control the development and function of moDCs (Chometon et al., 2020). They also affect the phenotype (appearance and function), immunological (activating immune responses), and tolerogenic potentials (inducing immune tolerance) (Chometon et al., 2020). Tolerogenic potentials refer to the ability of a substance or cell to induce a state of tolerance in the immune system. For example, a long culture period of moDCs may negatively affect the function by generating fewer immunogenic cells (Chometon et al., 2020). Some cells trigger an immune response, such as moDCs, while others suppress it, like Tregs (Wculek et al., 2020). *In vitro* generated moDCs are easier to obtain and modify than *in vivo* moDCs (Guinan & Lopez, 2020). This is because isolating and culturing *in vivo* moDCs is difficult, while *in vitro* methods are simpler and yield larger quantities (Guinan & Lopez, 2020). This accessibility allows researchers to modify *in vitro* moDCs in ways not possible with *in vivo* moDCs (Guinan & Lopez, 2020). These modifications can include expressing specific genes or targeting specific antigens (Guinan & Lopez, 2020). This approach facilitates studying moDCs role in various diseases and developing new

immunotherapies. However, *in vivo* moDCs are generally considered more efficient at presenting antigens compared to *in vitro* moDCs (Wculek et al., 2020).

2.4 Liposomes

Liposomes are derived from two Greek words "Lipos" meaning fat, and "Soma" meaning body (Anwekar et al., 2011). Liposomes are sphere-shaped vesicles with a range size of 0.025 μm to 2.5 μm (Gosavi & Salunkhe, 2020). The circulation half-life of liposomes depends on their composition and vesicle size (Nagalingam, 2017). The liposomes physical structure allows it to deliver both lipophilic drug molecules and its hydrophilic active products (Peyman et al., 2009). As previously mentioned, liposomes are made up of an aqueous core surrounded by one (Figure 2.5) or more bilayers of natural or synthetic lipids (Akbarzadeh et al., 2013a; Sercombe et al., 2015). The aqueous core and biocompatible lipid coating enable the delivery of a variety of macromolecules such as proteins, DNA, and imaging agents (Sercombe et al., 2015). The natural and synthetic phospholipids are capable of forming liposomes (Akbarzadeh et al., 2013a). Natural phospholipids and synthetic phospholipids excipients are contrasted in terms of manufacturing, use, and quality from the standpoint of industrial pharmaceutical technical advancement.

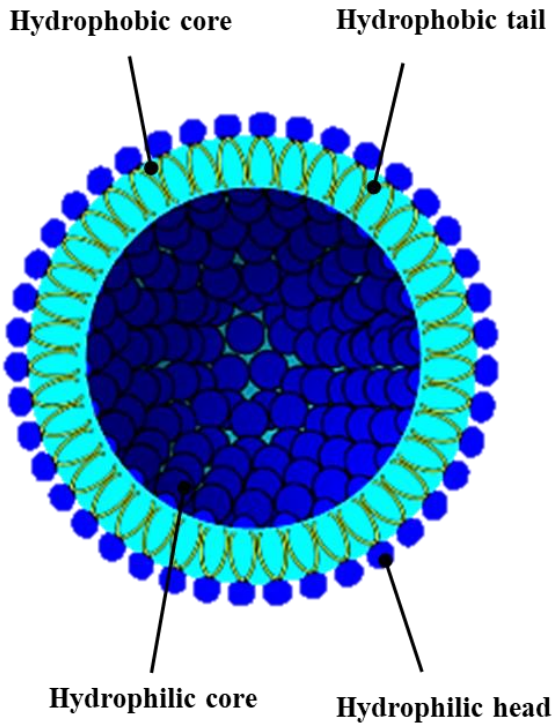


Figure 2.5 Schematic representation one layers of liposomes as a drug delivery system

Figure 2.6 shows hydrophobic drugs can be incorporated into the lipid bilayer, while hydrophilic drugs are typically encapsulated within the aqueous core. The encapsulation of macromolecules within liposomes enhances their stability and protects them from degradation by enzymes in the bloodstream (Natarajan et al., 2012). Liposomes are a cornerstone in the field of nanomedicine (Hosta-Rigau et al., 2014).

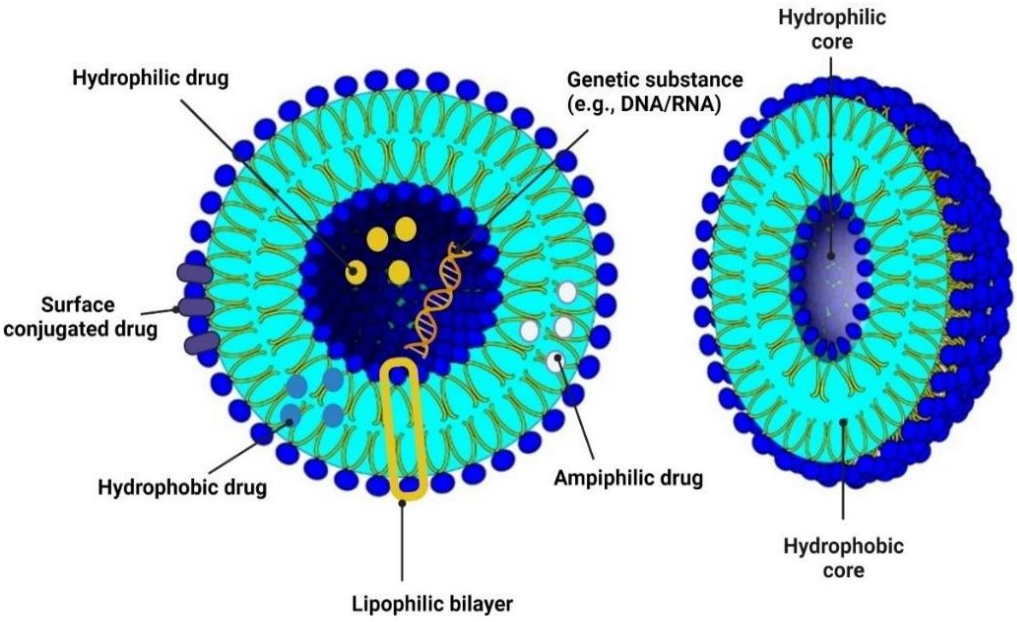


Figure 2.6 Schematic representation of liposomes bilayer as a drug delivery system. Liposomes is a sphere-shaped vesicle surrounded one or more bilayers of natural or synthetic lipids

2.4.1 The importance of liposomes in development as an adjuvant and delivery system

Liposomes are known as vaccine adjuvant delivery systems (VADS) to trigger immune responses against infectious disease (Khademi et al., 2018). The liposomal composition, size, surface charge, number of lamellae, rigidity of the bilayer, percentage of lipid to antigen ratio, and even the method of synthesis all have an impact on the adjuvant property of liposomes (Zamani et al., 2018). Modified liposomes give beneficial features of an effective VADS in delivering antigens, exhibit a strong biocompatibility and few adverse effects (N. Wang et al., 2019). The ability of liposomal adjuvants to stimulate a potent Th1 and Th17 immune response against infectious disease (Khademi et al., 2018).

Three reasons for liposomes as a carrier system are to enhance the immune response against protein subunit vaccines which is inherent immunogenicity, antigen