IMMUNOMODULATORY EFFECTS OF ASIATIC ACID AND MADECASSOSIDE OF *Centella asiatica* (PEGAGA) ON J774A.1 MOUSE MACROPHAGE CELL LINE

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by

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a	Significantly differences for combination groups when compared to single AA at their respective comparative doses ($p < 0.05$)
aa	Significantly differences for combination groups when compared to single AA at their respective comparative doses ($p < 0.01$)
aaa	Significantly differences for combination groups when compared to single AA at their respective comparative doses ($p < 0.001$)
aaaa	Significantly differences for combination groups when compared to single AA at their respective comparative doses ($p < 0.0001$)
b	Significantly differences for combination groups when compared to single MA at their respective comparative doses ($p < 0.05$)
bb	Significantly differences for combination groups when compared to single MA at their respective comparative doses ($p < 0.01$)
bbb	Significantly differences for combination groups when compared to single MA at their respective comparative doses ($p < 0.001$)
bbbb	Significantly differences for combination groups when compared to single MA at their respective comparative doses ($p < 0.0001$)
C1	Combination treatment (0.39+0.39) µg/mL
C4	Combination treatment (3.13+3.13) µg/mL
C7	Combination treatment (25+25) µg/mL
n	Size of sample from each experiment

*	Significantly difference when compared to untreated group ($p < 0.05$)
**	Significantly difference when compared to untreated group ($p < 0.01$)
***	Significantly difference when compared to untreated group ($p < 0.001$)
****	Significantly difference when compared to untreated group ($p < 0.0001$)
°	Degree
%	Percentage
X	Multiply
x	Times
<	Less than

LIST OF ABBREVIATIONS

AA	Asiatic acid
AA+MA	Asiatic acid and madecassoside
AIDS	Acquired immune deficiency syndrome
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
APCs	Antigen presenting cells
APS	Ammonium persulphate
ATCC	American type culture collection
β-actin	Beta actin
bp	Base pair
BW	Body weight
C	Celcius
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLR	C-type lectin receptor
cm	Centimeter
cm^2	Square centimeter
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DAMPs	Damage associated molecular patterns
dH_2O	Distilled water
DMEM	Dulbecco modified eagle's medium
DMSO	Dimethyl sulphoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DPX	Dibutylphthalate polystyrene xylene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal-regulated kinases 1
EtBr	Ethidium bromide
FBS	Fetal bovine serum
g	Gram
g	G force
GCCP	Good cell culture practice
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HGFs	Human gingival fibroblasts
HIV	Human immunodeficiency virus
HPLC	High performance of liquid chromatography
HRP	Horseradish peroxidase
IC_{50}	Inhibitory concentration 50
ID	Infectious disease
IDV	Intensity density value
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IκB-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells subunit alpha

IKK	IκBα kinase complex
IL	Interleukin
IL-1β	Interleukin-1-beta
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF 3	Interferon regulatory factor 3
JAK	Janus-kinase
JNK 1/2	C-Jun N-terminal kinases 1 and 2
kb	Kilo base
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium phosphate monobasic anhydrous
kg	Kilogram
L	Liter
LPS	Lipopolysacharides
M	Molar
MA	Madecassoside
MAL	MyD88-adapter-like
MAPkinases	Mitogen activated protein kinases
MHC	Major histocompatibility complex
MIC	Mean inhibitory concentration
mg	Miligram
µg	Microgram
MKK	MAPK kinase kinase
µL	Microliter

ml	Mililiter
mm	Milimeter
MRI	Mean relative intensity
mRNA	Mesengger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2-,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response 88
NA_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Natrium hydroxide
NED	*N*-(1-Naphthyl)ethylenediamine
NF-kβ	Nuclear factor kappa beta
ng	Nanogram
NK	Natural killer
NLR	Nucleotide-binding oligomerisation-like receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

pg	Picogram
PGE2	Prostaglandin E2
PI	Phagocytic index
P%	Phagocytosis percentage
PI-3K	Phosphoinositide 3-kinases
PMB	Polymyxin B
PRR	Pathogen recognition receptor
psi	Pounds per square inch
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RLR	Retinoic acid-inducible gene-I-like receptor
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
TAE	Tris acetate-EDTA
TAK 1	Transforming growth factor β-activated kinase 1
TBK 1	TANK-binding kinase 1
TBS	Tris buffer saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
Th1	T-helper 1

Th2	T-helper 2
TIRAP	TIR domain contain adaptor protein
THP-1	Human acute monocytic leukemia
TIR	Toll/interleukin-1 receptor-like
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Tpl 2	Tumor progression locus 2
TRAF 6	TNF receptor associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	Adapter-inducing interferon-β
U	Unit
UN	Untreated
UV	Ultra violet
VCAM-1	Vascular cell adhesion molecule 1
V	Volt
WHO	World Health Organisation

KESAN IMUNOMODULATORI ASID ASIATIK DAN MADEKASSOSID *Centella asiatica* (PEGAGA) TERHADAP TITISAN SEL MAKROFAJ MENCIT J774A.1

ABSTRAK

Makrofaj memainkan peranan penting dalam imuniti semulajadi bagi menghalang atau merawat badan daripada jangkitan. Jangkitan adalah ketaknormalan yang disebabkan oleh mikroorganisma dan pada masa sekarang, jangkitan ini di kawal oleh pelbagai ubat sintetik. Namun demikian, kebanyakan ubat sintetik yang sedia ada menunjukkan banyak kesan sampingan yang buruk dan menyebabkan kemunculan kerintangan terhadap agen anti-mikrob dalam kalangan mikroorganisma patogenik. Oleh itu, pencarian terhadap pelbagai agen alternatif yang mempunyai kebolehan untuk melakukan modulasi sistem imun perlu diterokai. Asid asiatik (AA) dan madekassosid (MA) merupakan dua komponen bioaktif utama pentasiklik triterpena yang diisolasi daripada *Centella asiatica* dan mempunyai potensi untuk melakukan modulasi pada sistem imuniti berdasarkan kajian yang lepas. Namun demikian, tiada data saintifik berkenaan kesan individu dan kombinasi kedua-dua komponen ini pada model sel titisan makrofaj mencit J774A.1 yang normal. Oleh yang demikian, kajian *in vitro* ini adalah untuk menguji hipotesis sama ada kedua-dua komponen ini berkebolehan untuk meningkatkan gerak balas imun semulajadi makrofaj. Secara ringkas, makrofaj di kultur dalam lima kumpulan yang terdiri daripada makrofaj yang tidak dirawat, makrofaj yang dirawat dengan lipopolisakarida sebagai kawalan positif, makrofaj yang dirawat dengan AA induk, makrofaj yang dirawat dengan MA induk dan makrofaj yang dirawat dengan

kombinasi AA dan MA (AA+MA) dalam masa 24 jam. Hasil dapatan kajian ini ditentukan mengunakan asai MTT, kaedah pemerhatian pada perubahan morfologi sel, tindak balas rantai polimerase transkriptase berbalik (RT-PCR) dan pemblotan Western. Selain itu, slaid pewarnaan Giemsa juga digunakan untuk menilai aktiviti fagositik. Asai "enzyme-linked immunosorbent" (ELISA) dan Griess juga dilaksanakan untuk menentukan penghasilan sitokin inflamatori dan nitrit oksida (NO) dalam supernatan makrofaj yang dirawat tersebut. Keputusan yang diperolehi menunjukkan rawatan induk MA dan kombinasi AA+MA tidak menunjukkan sebarang kesan sitotoksik yang signifikan terhadap makrofaj. Kombinasi rawatan AA+MA pada dos (25+25) μg/mL meningkatkan secara signifikan pengekspresan mRNA dan protein reseptor "toll like" 2 (TLR-2) dan TLR-4, aktiviti fagositosis, pengekspresan mRNA dan penghasilan sitokin inflamatori, penghasilan mediator iaitu rembesan NO dan pengekspresan sintase nitrit oksida teraruh (iNOS) serta pengekspresan semua protein yang terlibat di dalam laluan isyarat gerak balas inflamatori iaitu "myeloid differentiation primary response 88" (MyD88), "c-Jun N-terminal kinases 1 dan 2" (JNK 1/2), "extracellular signal-regulated kinases 1" (ERK 1/2) dan p38 apabila dibandingkan dengan makrofaj yang tidak dirawat serta makrofaj yang dirawat dengan setiap komponen induk AA dan MA (50 μg/mL) (p<0.0001). Kesimpulannya, penemuan kajian ini menunjukkan rawatan kombinasi AA+MA berkebolehan untuk meningkatkan gerak balas imun semulajadi makrofaj. Penemuan kajian ini mencadangkan pengambilan kedua-dua komponen ini sebagai makanan tambahan secara kerap akan sentiasa merangsang pengaktifan sistem imun bagi mencegah potensi jangkitan.

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ABSTRACT

Macrophage plays a vital role in innate immunity to prevent or treat the host from infection. Infection is an ailment which initiated by various pathogenic microorganisms and managed by many therapeutic synthetic drugs. However, almost available synthetic drugs presented with many adverse side effects and caused to the emergence of anti-microbial resistance among pathogenic microorganisms. Therefore, alternative agents that promote immunomodulatory activities needs to be explored. Asiatic acid (AA) and madecassoside (MA) are two main bioactive components of pentacyclic triterpenes isolated from *Centella asiatica* have this immunomodulatory potential based on the previous studies. However, there is no scientific data related to the effects of these individual and combination compounds on normal model of J774A.1 mouse macrophage cell line. Therefore, this *in vitro* study tested the hyphothesis whether both compounds are able to elicit an innate immune function of macrophages. Briefly, macrophages were cultured in five groups; untreated macrophage, macrophage-treated with lipopolysaccharides as a positive control, macrophage-treated with single AA, macrophage-treated with single MA and macrophage-treated with combination AA and MA (AA+MA) for 24 hours. The immunomodulatory effects of these compounds were determined using MTT assay, an observational method for cells morphological changes, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis. In addition, Giemsa staining was also used to assess the phagocytic activity while

enzyme-linked immunosorbent assay (ELISA) and Griess assay were used to determine the production of pro-inflammatory cytokines and nitric oxide (NO) in culture supernatants of treated macrophages, respectively. The results showed both single MA and combination treatments of AA+MA did not exert any cytotoxic effects on macrophages. Furthermore, the combination treatments of AA+MA at a dosage of (25+25) μg/mL were significantly enhanced the mRNAs and proteins expression of TLR-2 and TLR-4 receptors, phagocytosis activity, mRNAs expression and production of pro-inflammatory cytokines, production of mediators; NO secretion and inducible nitric oxide synthase (iNOS) expression as well as expression of proteins involved in the signalling pathway of inflammatory response including myeloid differentiation primary response 88 (MyD88), c-Jun N-terminal kinases 1 and 2 (JNK 1/2), extracellular signal-regulated kinases 1 (ERK 1/2) and p38 as compared to the untreated macrophages and each single compound of AA and MA-treated macrophages (50 μg/mL) (p<0.0001). In summary, the findings suggest that the combination treatments of AA+MA are able to upsurge innate immune responses of macrophages. The findings of this study suggest that the regular consumption of these compounds as a food suplement will continuously stimulate the immune system to protect against potential infections.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The immune system which comprises of innate and adaptive immunity plays a crucial role in human health as it protects the body from invading pathogens and treats existing diseases (Konradt and Hunter, 2018). Resultantly, a minor infection can already trigger severe disease in the host if it has defected or fragile immune system (Spoor *et al.,* 2019). Therefore, it is believed that the development of abnormal conditions such as infection and cancer are related to the alteration and the weakness of the host's immune system (Fleming-Davies *et al.*, 2018; Weksler and Lu, 2014). One of the ways to reduce this problem is to modify the host's immune responses by increasing the competency of this system in preventing or eliminating the aetiological agents that cause diseases (Aspinall and Lang, 2018). As a consequence, the modification of immune responses that function to increase or lower the immune alertness which is also known as immunomodulation to treat diseases through potential agents has been a great discovery to explore (Hadden, 1987). As an example, the modulation of primary or innate immunity has a major influence on the host's capability to react rapidly and effectively to a diverse group of pathogens that caused diseases (Thakur *et al.,* 2019).

In order to acquire a better understanding of the immunomodulatory effects of potential agents such as plant compounds, the researcher must consider its effects on the immune cells which function to regulate immunity in a body. One of the most

important cells is macrophage which is a key player and the main component of innate immunity that is involved in the initial protection against pathogenic microorganisms that encounter the host (Byrne *et al.,* 2015; Weiss and Schaible, 2015). Resident macrophages always present in the tissue such as gastrointestinal tract (Grainger *et al.,* 2017), lung (Gordon and Read, 2002), liver, bone and skin (Davies *et al.,* 2013). The functions of specific pathogen recognition receptor (PRR) expresses on the cell surface of macrophage are to recognise and interact with the various components of a pathogen associated molecular pattern (PAMP) on microorganisms. These processes activate the macrophage and enhance its responses towards stimuli (Leavy, 2015).

Additionally, activated macrophage ingests microorganisms into vesicles through a specific process called phagocytosis (Masud *et al.*, 2019). Subsequently, stimulated macrophage plays a key role in the primary immune responses by producing various pro-inflammatory cytokines including interleukins and chemokine to eradicate infectious agents and tumour (Moghaddam *et al.,* 2018). The production of these substances promotes further cellular reactions of innate immunity that serve to kill and degrade the pathogenic microorganisms that have encountered the host (Duque and Descoteaux, 2014). Tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1-beta (IL-1β), IL-12 and IL-6 are the secreted pro-inflammatory cytokines involved in host protection against infection (Nonnenmacher and Hiller, 2018). Pro-inflammatory cytokines also stimulate macrophage to produce toxic reactive nitric and oxygen species that consist of nitric oxide (NO) and hydrogen peroxide (H_2O_2) for intracellular killing (Nathan and Hibbs, 1991; Trujillo and Radi, 2019). Activated

macrophage is also plays a role as an antigen presenting cells to stimulate T helper lymphocyte in adaptive immune response (Roche and Furuta, 2015).

Activation of innate immune responses of macrophage towards pathogens is also regulated by the intracellular inflammatory signalling pathway (Newton and Dixit, (2012). Initially, the interaction of PAMP with toll-like receptor (TLR) which is a part of PRR promotes downstream inflammatory signalling pathways via myeloid differentiation primary response 88 (MyD88) adaptor proteins. This receptor-ligand interaction leads to the activation of mitogen-activated protein (MAP) kinases involved in this pathway and further promotes the phosphorylation of MAPkinases activator protein-1 (AP-1) (Vidya *et al.*, 2017). MAPkinases consist of extracellular signal-regulated protein kinase 1/2 (ERK 1/2), c-Jun N-terminal kinases 1/2 (JNK 1/2) and p38 MAP kinases (p38) which regulate various cellular functions differentially including activation of macrophage (Arthur and Ley, 2013). On top of that, AP-1 is a transcription factor that coordinates the expression of genes that encode inducible nitric oxide synthase (iNOS), IL-12p40, IL-1β and TNF-α (Lloberas *et al.*, 2016). iNOS catalyses the generation of NO, a molecule secreted by macrophage which plays a key role in the innate immune responses (Bogdan, 2015).

Infectious disease (ID) is caused by various pathogenic microorganisms consist of bacteria, viruses, parasites and fungus (Krumkamp *et al.*, 2015). The severity of ID is closely related to the capability of the host immune response to resolve the infections. Hence, it influences the ability of pathogen to establish its existence in a host (Sorci *et al.*, 2013). This consequence is related to the finding by Li *et al.*, (2016) suggested that the pattern of infectious disease was related to the decrease of

immunity and malnutrition in younger and elderly groups which led them susceptible to infections, increased the severity and facing poor progress after treatment. Presently, public awareness regarding ID has been increased due to various factors such as rapid spread of diseases, high incidence of morbidity and mortality and slow development of vaccines. Economically, the management of ID is very costly (Cunningham *et al.,* 2017). The common current clinical therapies for management of ID are mainly based on the elimination of the aetiologic pathogen that triggers the diseases and to release the symptoms by using synthetic medicinal drugs (Shane *et al.*, 2017). However, the synthetic drugs were caused several adverse side effects and emergence of anti-microbial resistance among the pathogenic microorganisms (Dyson *et al.,* 2019). According to World Health Organisation (WHO), the occurrence of anti-microbial resistance is growing in both hospitalised patients and community and depends on the effectiveness of anti-microbial agents as well as the spread of resistant pathogens among patients (WHO, 2000).

The use of natural products and their derivatives as a source of immunomodulation agents has become increasingly important nowadays due to the adverse side effects of the drugs used in the clinical setting. Immunostimulants from natural products as a potential agents are considered beneficial to prevent or treat bacterial and viral infections (Chauhan *et al.,* 2014; Park *et al.,* 2018) as well as in cancer (Park *et al.,* 2013) which is caused from immunodeficiency conditions.

Asiatic acid (AA) and madecassoside (MA), two important bioactive pentacyclic triterpenes which derived from *Centella asiatica* that contribute to the various valuable medicinal properties of this plant (Mahmood *et al.,* 2016). Based on

the previous studies, these compounds possesses a wide range of biological functions including anti-microbial (Idris and Nadzir, 2017), immunomodulatory (Jayathirtha and Mishra, 2004), anti-inflammatory, anti-oxidant (Nurlaily *et al.,* 2012) and anti-cancer activities (Zhang *et al.,* 2013). Instead of being majorly found in *C. asiatica*, AA also can be isolated from other plants such as from *Salvia miltiorrhiza* (Tung *et al.,* 2017), *Psidium guajava* (Anand *et al.*, 2020), *Punica granatum* (Arun and Singh, 2012), *Averrhoa carambola*, *Gynura bicolor* and *Brassica juncea* (Yin, 2015). However, based on the literature, AA from *C. asiatica* has gained more interest among researcher to study for its pharmacological activities. Therefore, in the present study, AA and MA from *C. asiatica* were selected and tested in single and combination treatments on mouse macrophage cell line to evaluate their immunomodulatory effects on innate immune functions of this cell.

1.2 Problem Statement

ID receives the highest concern among healthcare providing organisation, mainly in developing countries (WHO, 2000). It is mainly managed through preventive measures including vaccination (Maslow, 2017) as well as using suitable synthetic medicinal drugs such as antibiotics and anti-viral to treat the existing diseases (Bekerman and Einav, 2015). However, antibiotic has many serious adverse effects such as allergic reactions and removal of normal flora bacteria in the body (Glick, 2016). Additionally, Tamma *et al.,* (2017) also had stated that there were adverse conditions such as nausea, vomiting, anaphylaxis, hematologic and neurological abnormalities within 30 days of antibiotic initiation among hospitalised patients. Furthermore, there is also antibiotic resistance among enteric pathogens and it is a common cause of gastrointestinal infection in human. Research finding by

Shields *et al.*, (2016) showed that 30 % of resistant cases had been detected among carbapenem-resistant Enterobacteriaceae infected patients which exhibited re-current infections after consuming ceftazidime-avibactam drug. Likewise, the consumption of this drug also promotes acute kidney injury which was also detected in three out of 31 patients within seven days prior to treatment.

Thus, other alternative for immunomodulatory agents from other sources such as natural products receive more interest among researchers. It is because the specific agents have the potentials to elicit the normal body's immune defence system to provide improved protection against microbial infection. Besides, immunomodulatory therapies also provide benefits by targeting the host than the specific pathogen which later would reduce the incidence of microbial resistance (Hancock *et al.*, 2012).

1.3 Rationale of Study

There is a large cluster of various bioactive compounds with significant amounts of pharmacological activities originated from medicinal floras and herbs. These beneficial compounds which contained specific bioactivities are beneficial in promoting human health. In 2013, 1453 new bioactive compounds have been patented from natural products approved by the US Food and Drug Administration (Katz and Baltz, 2016). They have been widely used as health supplements, nutritive products and medications since pre-historic time. Natural product-based drugs promote the significant role in the pharmaceutical industry. Lately, safe drugs with low side effects and enclosed with chemical molecules that act as selective ligands to

effectively prevent and cure diseases have become a mission for drug development programs (David *et al.,* 2015).

In Malaysia, *C. asiatica* is regularly consumed either in the form of products or eaten raw as a salad by a general healthy population with a lack of scientific evidence related to the immunomodulatory activities of this plant in healthy experimental models. Thus, it is believed that *C. asiatica* has a potential to modulate the innate immune responses of resident macrophages in a body which acts as a prevention against pathogenic microorganisms that can cause numerous ID. Expecting the potential of AA and MA as the major compounds in *C. asiatica* that able to modulate the immune responses in the previous scientific investigations, they have been chosen as the main interest in this study. As far as our literature survey could ascertain, there has been no scientific data related to the effects of these individual and combination compounds on the normal model of mouse macrophage cell line (J774A.1). Hence, by using this model, the present study was conducted to determine the effects of single AA, MA and their combination (AA+MA) on the innate immune responses of macrophages. Hopefully, this study will provide initial knowledge in the development of a novel preventive agent to specifically enhance the innate immune responses of the host and presents an ideal strategy for preparation and protection of the host against invasion of various agents that can cause ID rather than treating existing diseases.

1.4 Objectives of Study

1.4.1 General objective

To evaluate the immunomodulatory activities of asiatic acid (AA) and madecassoside (MA) derived from *Centella asiatica* on the innate immune responses of J774A.1 mouse macrophage cell line.

1.4.2 Specific objectives

1. To evaluate the cytotoxicity of individual and combination of AA and MA treatments on J774A.1 mouse macrophage cell line by determining the viability percentage and morphological changes.

2. To evaluate the effects of individual and combination of AA and MA treatments on innate immune responses of J774A.1 mouse macrophage cell line.
    i. To determine the messenger ribonucleic acids (mRNAs) expression of TLR-2, TLR-4 and their proteins expression.
    ii. To determine the phagocytosis activities, the production of NO and expression of iNOS protein.
    iii. To determine the mRNAs expression of pro-inflammatory cytokines (IL-1β, IL-12p40, TNF-α) and their proteins production.

3. To evaluate the effects of individual and combination of AA and MA treatments on the expression of MyD88 protein and MAPkinases (ERK 1/2, JNK 1/2 and p38) that involved in the inflammatory signalling pathway in J774A.1 mouse macrophage cell line.

1.5 Hypotheses

The hypotheses of this study are:

1. The individual and combination treatments of AA and MA show non-cytotoxic on J774A.1 mouse macrophages cell line.
2. Combination treatments of AA and MA increase all the innate immune responses of J774A.1 mouse macrophages cell line when compared to the untreated and individual groups.
3. Combination treatments of AA and MA enhance the expression of targeted proteins of J774A.1 mouse macrophages cell line involved in MAPkinases signalling pathway when compared to the untreated and individual groups.

1.6 Flowchart of the Study

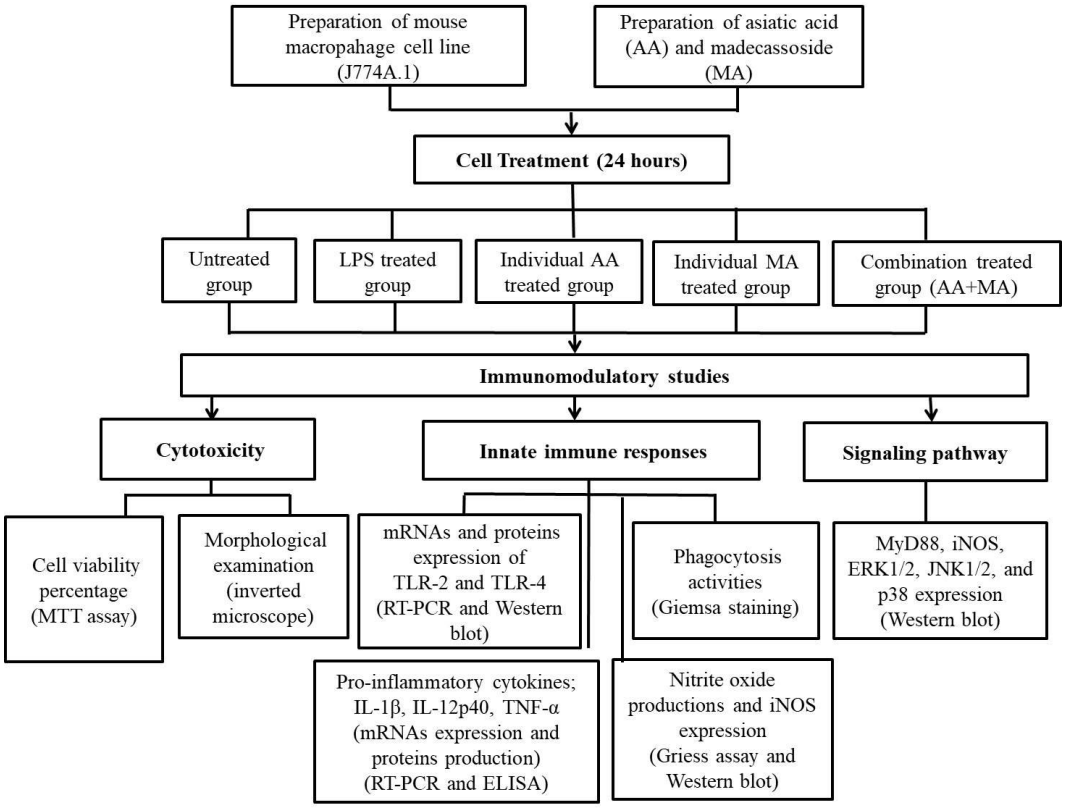

Figure 1.1 Flow chart of research activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Immune Systems

An immune system consists of a group of cells, molecules and tissues that facilitate the resistance to infections (Delves and Roitt, 2000). The important function of the immune system is to prevent from future infections (Keller and Stiehm, 2000) and to eliminate established infections which are already presented in the host (Weiss and Schaible, 2015). There are other roles of the immune system including recognising and responding to newly introduced proteins and tissue grafts which are important in transplantation (Zeiser *et al.*, 2019). In addition, the immune system also plays a crucial role in host protection against tumor as it has postive potential in cancer immunotherapy (Yang, 2015).

The mammalian defence systems are divided into two which are innate immunity and adaptive immunity as showed in Figure 2.1. The anatomic and physiological barriers, such as intact skin, special lysozyme; saliva and tears as well as acidic pH of the stomach offer the initial line of protection against diverse pathogens (Turvey and Broide, 2010). Innate which is also known as primary or natural immunity involves specific immune cells that mediates the first protection to human body, quick respond to pathogens and boost the protection offered by the natural immune barriers (Lambrecht and Hammad, 2014). On the other hand, macrophage and dendritic cell have a distinct role as an antigen-presenting cells in adaptive immunity (Hespel and Moser, 2012; Weiss and Schaible, 2015). In brief,

adaptive immunity consisting of B and T lymphocytes that facilitate later actions as a response to infections which able to escape from innate immunity (Iwasaki and Medzhitov, 2015).

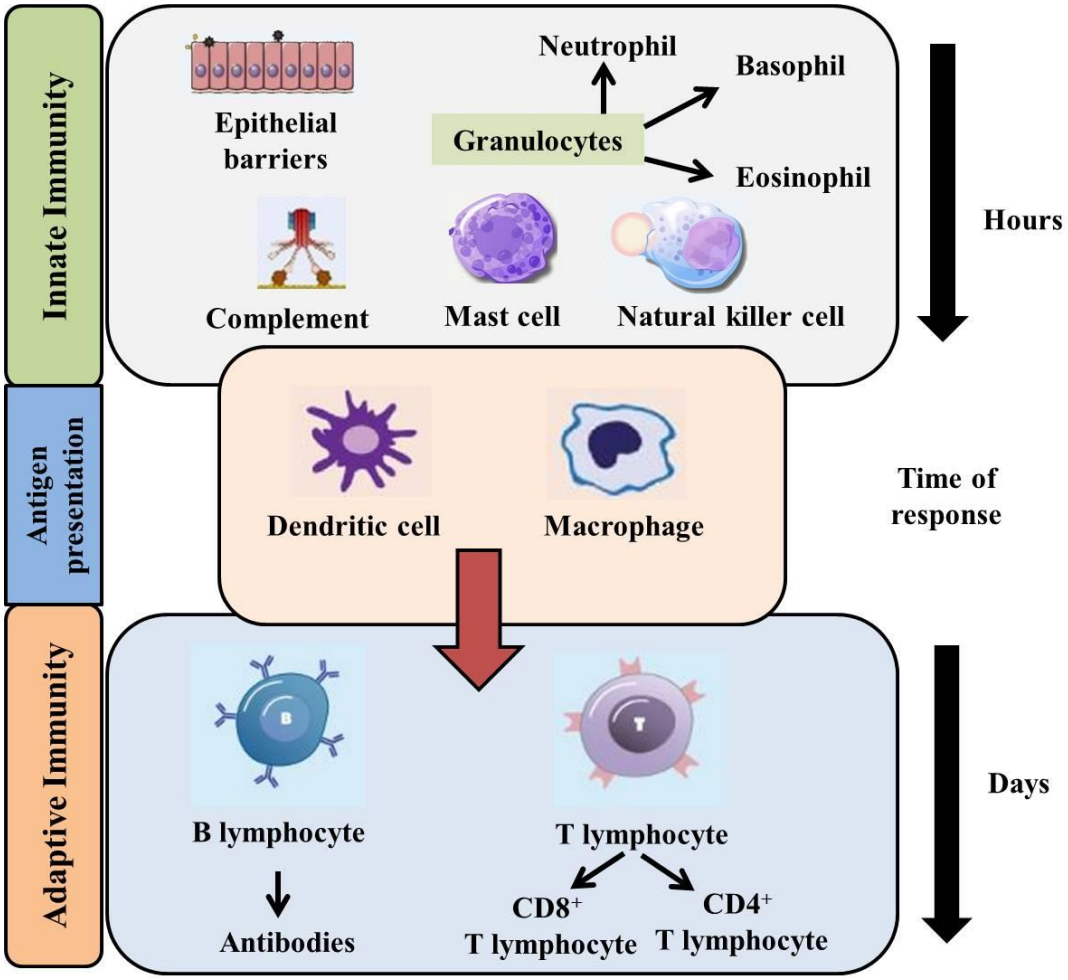

Figure 2.1 Cellular components of the mammalian immune system. Modified from Nicholson (2016) and Yamauchi and Moroishi (2019).

2.1.1 Innate immunity defence

Innate immunity is a natural task performed especially by hematopoietic-originated cells including macrophage, neutrophil, dendritic, eosinophil, and natural killer (NK) cell. This type of defence depends mainly on the cells that able to recognise microbes, move towards them, picking them by phagocytosis and kill them (Spiering, 2015). Likewise, there are also significant roles of non-hematopoietic originated cells that consist of specialised epithelial cells barriers that function in the early immune response towards encountered pathogens (Turvey and Broide, 2010). For instance, the gut barrier is the largest component compare to others and particularly adaptable to colonisation by gut microbiota. Its function is to help in digestion and contributes to the improvement and role of the mucosal immune system (Ahluwalia *et al.,* 2017). However, the disruption of the barriers caused by a pathogenic microorganism leads to infection in the gastrointestinal tract (Peterson and Artis, 2014).

The dynamic mechanism of innate immune responses towards microbial infection and tissue injury is known as inflammation. The effective inflammatory responses provide a wide-ranging protection against infections and later coordinate long-standing to acquire immunity toward specific pathogens (Xiao, 2017). A normal inflammatory response involves four components; inflammatory triggers, detection receptors, inflammatory cytokines and mediators which are regulated by different inflammatory pathways (Medzhitov, 2010). The initial immune response is crucial for early pathogen recognition which is related to genetically predetermined germline-encoded receptors on their immune cells known as pathogen recognition receptor (PRR) (Tartey and Takeuchi, 2017). These non-specific receptors recognise

either conserved structures expressed by various classes of microbes or other molecules released during infection. There are many microbial ligands originated from structural components of bacteria, fungi, viruses and other biosynthetic molecules such as nucleic acids that can be recognised by PRR and further activate the immune cells like macrophage and dendritic cell (Brubaker *et al.,* 2015). As a result, cell activation promotes stimulation of innate immune responses through the process of opsonisation and phagocytosis as an initial host protection against infection (Brubaker *et al*. 2015; Kawai and Akira, 2010).

Additionally, during phagocytosis the involved immune cells also produce numerous pro-inflammatory cytokines such as TNF and IL-1 that function to recruit and activate other intravascular leukocytes (Carrero *et al.,* 2012) as well as to stimulate the maturation of dendritic cells to further enhance the adaptive immune responses (Steinbach and Plevy, 2015). These cytokines also function to vasodilate the local blood vessel at the targeted tissues for neutrophils migration to the site of infection which involve a multistep process (Fink and Campbell, 2018). Besides, TNF and IL-1 also stimulate the endothelium of small vessels at the site of infection to rapidly express two adhesion molecules called as P-selectin and E-selectin which act as a ligand for integrin and chemokines (Konradt and Hunter, 2018). The tethering and rolling of blood neutrophils on the endothelium are mediated by both adhesion molecules (Gong *et al.,* 2017).

Neutrophils activation and their migration via endothelium to the site of infection also activated by chemokine (Turner *et al.,* 2014). Chemokine constitute a large family of low molecular-weight of cytokine which is secreted in response to pathogens and other inflammatory stimuli. The production of chemokine is regulated

by NO and prostaglandin (Kobayashi, 2010). The high concentration of chemokine bound on the luminal surface of endothelial cells displayed to the leukocytes. This chemokine enhances the motility of leukocytes and their integrin's affinity to the ligands on the endothelium Consequently, leukocytes start to migrate along the chemokine concentration gradient and perform diapedesis between endothelial cell wall to the site of infection (Pilar *et al.,* 2017). Meanwhile, IL-12 functions to stimulate T helper lymphocytes and promotes cell-mediated immunity to combat the pathogens and cancer that established in a host (Duque and Descoteaux, 2014).

At the site of infection, neutrophils undergo apoptosis after performing roles to kill pathogens. Meanwhile, monocytes evolve into macrophages. The apoptotic neutrophils are then cleared by macrophages to resolve the inflammation (Newton, and Dixit, 2012). Besides, the production of pro-inflammatory cytokines also stimulates the macrophages and dendritic cells to destroy the phagocytosed pathogens through intracellular killing by releasing toxic substances (Duque and Descoteaux, 2014; Forrester *et al.,* 2018; Mosser and Edwards, 2008). Meanwhile, eosinophil, basophil and mast cell are essential components of allergic inflammation (Stone *et al.,* 2010). Besides, resident eosinophils in tissue are specifically involved in host responses against helminth infection (Weller and Spencer, 2017).

Unfortunately, there are times when pathogens are able to rapidly multiply in a host and undergo a revolution to escape the defence mechanisms in the innate immune system (French *et al.,* 2004). Nevertheless, the sophisticated and efficiency of the innate immune system have been developed further to identify the microbial components and link them to adaptive immunity. Therefore, this prevents the severe

pathogenesis caused by pathogens in a host (Thimme *et al.*, 2006; Turvey and Broide, 2010).

2.1.1 (a) Macrophage

Macrophage has gained a great interest within the previous decade and currently their role in the stimulation of innate immunity to prevent ID is getting appreciated (Schepetkin and Quinn, 2006). Macrophage or mononuclear phagocyte is majorly found in connective tissues and every organ in the body (Epelman *et al.*, 2014) and this cell also widely known as professional phagocyte in which it expresses a multitude of receptors on its surfaces (Murray and Wynn, 2012). Normally, the size of peritoneal macrophage is about 10 to 30 µm in diameter and its cytoplasm contains basophilic vacuoles and ovoid nucleus (6 to 12 µm in diameter). Peritoneal macrophage contains dark gray rod-shaped mitochondria and light gray diffuse cytoplasm that can be observed via phase contrast microscopy. Meanwhile, the appearance of vacuoles and granules is influenced by physiological state of the macrophage (Elhelu, 1983).

Macrophage is derived from precursor cells in the bone marrow that develop into monocyte in the peripheral blood before being matured, entering and residing in the specific tissues in the human body (Mass, 2018). Stem cells of the granulocytic–monocytic lineage in the bone marrow that exposed to cytokines such as the granulocyte macrophage colony-stimulating factor and IL-3 stimulate the production of monocytes (Kumar and Bhoi, 2017). The monocyte presented by approximately 5 to 10 % of leukocytes in peripheral blood and its size and nuclear morphology is

varied and has dissimilar amounts of granularity (Gordon and Taylor, 2005). The macrophage that involve in a regulation of inflammatory responses is known as M1 macrophage while M2-type macrophage reduces this activity and increase the tissue healing process (Liu *et al.,* 2014).

There are various homeostatic functions of tissue macrophage and depending on its location in a body (Ginhoux and Jung, 2014). For instance, macrophage reside in the intestinal and colon of gastrointestinal tract which is the major population of mononuclear phagocyte in the body (Hine and Loke, 2019) performs an essential role in defence and homeostasis in intestinal circulation system (Grainger *et al.,* 2017). The bacteria and antigens that breach the epithelial barrier activate the gut macrophages to regulate inflammatory responses as a protection against harmful pathogenic microorganisms (Smith *et al.,* 2011) as well as to eliminate foreign debris and dead cells (Hirayama and Iida, 2018).

The resident macrophage recognise the infectious microbes via interaction of TLR-PAMP (Zhou *et al.,* 2016) and complement receptor-opsonised pathogen (Bohlson *et al.,* 2014). The interaction initiates the phagocytosis process which leads to the enhancement of transcription factors involved in the expression of genes encoding specific enzymes, proteins and pro-inflammatory cytokines. All the signalling molecules are involved in the anti-microbial tasks of activated macrophage (Kawasaki and Kawai, 2014). An activation of macrophage in enhancing phagocytic and microbicidal activities is the main element in host's initial immune responses to a diverse array of pathogens. This stage is very important to prevent the host from infection or to control the spread of invading pathogens (Mosser and Edwards,

2008). An active macrophage also performs antigen presentation to the adaptive immune system (Arnold *et al.,* 2015). Moreover, macrophage also contributes to the degradation of apoptotic cells and neoplastic cells (Grainger *et al.,* 2017).

There are numerous macrophage-like cell lines that have been developed for *in vitro* study such as cytotoxicity assessment, mechanisme of surface receptors, anti-microbial, anti-cancer and immunomodulatory (Paradkar *et al.,* 2017). Based on Saleh and Bryant (2018), the benefit of using cell line is it is ready to use for testing as well as having high stability in culture. Murine macrophage (J774A.1) that used in this study is a secondary transformed and immortalised cell line (Chamberlain *et al.,* 2015). This cell is always involved in initial testing and screening of plant extracts and bioactive compounds on the immune parameters before proceeding to animal and clinical studies (Chalons *et al.,* 2018; Machado *et al.,* 2019; More and Pai, 2011; Szliszka *et al.,* 2013).

2.1.1 (b) Pathogen recognition receptor (PRR)

Presently, four different groups of PRR families have been identified which are TLR, nucleotide-binding oligomerisation-like receptor (NLR), C-type lectins receptors (CLR), and retinoic acid-inducible gene (RIG)-I-like receptor (RLR) (Dowling and Mansell, 2016; Karin and Meylan, 2006; Takeuchi and Akira, 2010). Among other classes of PRR, the TLR group is one of the most important PRR families for rapid detection of invading intracellular and extracellular pathogens (Aderem and Ulevitch, 2011) and have been widely studied (Dowling and Mansell, 2016; Mogensen, 2009). This receptor acts as the first responded molecules in innate immunity to the existence of the pathogens in a host (Akira and Hemmi, 2003).

The role of TLR in innate immune defence was first found in the Drosophila model (Vogel, 2012). Mammalian TLR consists of 13 members as presented in Table 2.1. Ten TLRs (TLR-1 to TLR-10) have been identified in a human and 13 (TLR-1 to TLR-13) in a mouse (Pandey *et al.*, 2014). TLR functions to recognise a variety of microbial ligands known as PAMP (Akira and Hemmi, 2003) or damage associated molecular pattern (DAMP) (Tang *et al.,* 2012). Cellular stress or tissue damage caused host cells to release endogenous molecules such as heat shock protein which present the most components of DAMPs (Kataoka *et al.,* 2014). Meanwhile, PAMPs are molecules shared by the microorganism of the same type that consist of lipopolysaccharides (LPS) (Ranf, 2016), bacterial lipoteichoic acids (Morath *et al.,* 2003), lipopeptides (Takeda *et al.,* 2002), glycolipids (Schick *et al.,* 2017), flagellin and zymosan (Aakanksha *et al.,* 2018) which have always been targeted by host's innate immune responses.

Some of TLRs exist in the endosome to recognise the ingested microbes (Hemmi *et al.,* 2002; Lester and Li, 2014) and other TLRs are located on the surface of the cell membrane to recognise products of extracellular microbes (Ryan *et al.,* 2011). Then, these recognitions promote TLR-PAMP binding and activate the macrophages to enhance innate immune responses in defending the host against infection (Brubaker *et al.,* 2015). The function of TLR is important to defense the host against pathogens (Savva and Roger, 2013) by stimulating transcription factors which further stimulate innate and adaptive immune responses (Peralta *et al.,* 2007; Smale, 2015; Takeda & Akira, 2004). In detail, the activation of these transcription factors stimulates the secretion of numerous pro-inflammatory cytokines, interferons

and mediators (Muzio *et al.,* 2000). Besides, opsonisation and phagocytosis activities also enhance in response to the TLR activation (Brubaker *et al.,* 2015). All these activities increase innate immune responses of macrophage and further promote its anti-microbial responses to prevent the spread of an early infection through stimulation of the specific inflammatory signalling pathway (Brightbill and Modlin, 2000).

Although there are several TLR expressed by innate immune cells, TLR-2 and TLR-4 are immensely significant and have gained much interest due to their capability to recognise diverse molecular patterns of pathogens that include bacteria, viruses, fungi and protozoa (Mukherjee *et al.,* 2016). Thus, the present research would determine the effects of targeted compounds on the TLR-2 and TLR-4 expression of J774A.1 mouse macrophage cell line.

Table 2.1 TLRs and their ligands (Behzadi and Behzadi, 2016; Mukherjee *et al.*,2016; Pandey *et al.*, 2014; Takeuchi and Akira, 2010; Satoh and Akira, 2016).

Type of TLR	**Site**	**Ligand**	**Source of the ligand**
TLR-1	Cell membrane	Triacyl lipoprotein	Bacteria
TLR-2	Cell membrane	Lipoprotein,lipoteichoic acid, glycolipids, zymosan, LPS	Bacteria, viruses, parasites, fungi, protozoa, self
TLR-3	Endosome	dsRNA	Viruses
TLR-4	Cell membrane	LPS and P fimbriae	Bacteria, viruses, fungi, protozoa
TLR-5	Cell membrane	Flagelin	Bacteria
TLR-6	Cell membrane	Diacyl lipoprotein	Bacteria, viruses
TLR-7	Endosome	ssRNA	Viruses, bacteria, self
TLR-8	Endosome	ssRNA	Viruses
TLR-9	Endosome	CpG-DNA	Viruses, bacteria,protozoa, self
TLR-10	Endosome	Triacyl lipoprotein and diacyl lipoprotein	Bacteria, viruses
TLR-11	Endosome	Profilin-like molecules	Protozoa, parasites
TLR-12	Endosome	Profilin-like molecules	Parasites
TLR-13	Endosome	23s rRNA	Bacteria

2.1.1 (b)(i) Toll-like receptor-2 (TLR-2)

TLR-2 presence on the cell membrane of immune cells like macrophage and has a role to recognise common PAMPs of various microorganisms such as lipoteichoic acid (Cox *et al.,* 2007), peptidoglycan (Liu *et al.,* 2001), lipoproteins (Ihalin and Asikainen, 2018) and zymosan (Underhill and Ozinsky, 2002b) which then activates the signalling pathway of an inflammatory response. Besides that, TLR-2 is also able to detect certain structural variations of LPS such as those derived from *Porphyromonas gingivalis* and *Leptospira interrogans* (Underhill and Ozinsky, 2002b). TLR-1 and TLR-6 are separately connected with TLR-2 through heterodimers and each combination acts differently to identify PAMPs of microorganism (Wetzler, 2003). For example, glycosylphosphatidylinositol which contains three fatty acid components of *Plasmodium falciparum* activates macrophage effectively through TLR-2/TLR-1 to mediate inflammatory response (Zhu *et al.,* 2011). Meanwhile, MyD88/NF-κB signalling pathway was shown to stimulate through the interaction of TLR-2/TLR-6 with lactic acid bacteria (Ren *et al.,* 2016).

Based on a review study by Mukherjee *et al.,* (2016), several bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and Wolbachia induced pro-inflammatory cytokines such as TNF-α, IL-12, IL-1 by activation of TLR-2. The other cytokines like IL-17 and IL-22 are also secreted through the activation of TLR-2/TLR-1 (Nishimori *et al.,* 2012) while the stimulation of TLR-2/TLR-6 activates the release of IL-10 (Ren *et al.,* 2016). The interaction of TLR-2 with microbial ligands stimulates the cytokine production of macrophage via MyD88-dependent pathway which amplifies MAPkinases (Rojas *et al.,* 2014), NF-kβ (Qin *et*

al., 2016), and phosphoinositide 3-kinases (PI-3K) activations (Lasunskaia *et al.,* 2006).

2.1.1 (b) (ii) Toll-like receptor-4 (TLR-4)

TLR-4 recognises mainly Gram negative bacterial LPS (Lu *et al.,* 2008) as well as other components of pathogens such as teichuronic acid of Gram-positive bacteria (Yang *et al.,* 2001) mannuronic acid polymers of Gram-negative bacteria (Flo *et al.,* 2002) and F protein from respiratory virus (Kurt *et al.,* 2000). Additionally, endogenous molecule such as hyaluronic acid (Ferrandez *et al.,* 2018), β-defensin (Feng *et al.,* 2017) and heat shock proteins (Rosenberger *et al.,* 2015) are also able to interact either directly or indirectly with TLR-4.

Similar with TLR-2, the interaction of PAMP with TLR-4 receptor leads to the activation of their intracellular domain of Toll/interleukin-1 receptor-like (TIR) which cause conformational changes in this molecule (Muzio *et al.,* 2000). Then, the TIR domain recruits either TIR-domain-containing adapter myeloid differentiation factor 88 (MyD88) and MyD88-adapter-like (MAL) which also known as TIR domain contain adaptor protein (TIRAP) which involved in MyD88 dependent pathway or TIR adapter-inducing interferon-β (TRIF) and TRIF-related adapter molecule (TRAM) (MyD88-independent pathway) (Molteni *et al.,* 2016). The activation of MyD88-dependent pathway leads to the activation of both MAPkinases (Gupta *et al.,* 2017) and nuclear factor kappa beta (NF-κβ) (Tripathi and Aggarwal, 2006) and further promotes the synthesis of pro-inflammatory cytokines and chemokines. In contrast, the type I interferon is induced through activation of MyD88-independent pathway (Yamamoto *et al.,* 2003) and depends on the

endocyotosis of TLR-4 which requires the presence of CD14 upon microbial detection (Zanoni *et al.*, 2011).

2.1.1(c) Phagocytosis

Phagocytosis is defined as the engulfment of particles with more than 0.5 µm in diameter by microorganisms or phagocyte cells. This functional process is important for innate immunity and involve numerous signalling pathways (Richards and Endres, 2016). Eli Metchnikov, the Father of Innate Immunity, was the first to discover the fact that the phagocytic activity of amoeboid cells was related to the host defence (Gordon, 2016). Later, the typical models of microbe-innate immune interactions were developed for *in vitro* and *in vivo* investigations to acquire a better understanding regarding this interaction (Tauber, 2003). Phagocytosis is a complex process which consisting of uptake, digestion and removal of pathogens and apoptotic cells which are important for host defence and tissue homeostasis (Rosales and Uribe, 2017).

The phagocytosis process of macrophage is illustrated in Figure 2.2. In brief, after binding the specific TLR of macrophage or complement-coated particles with PAMP, the phagocytosis process is continued with pathogens that are surrounded by membrane protrusion, ingested into membrane-bound vesicle called phagosomes which then is fused with lysosomes to make up phagolysosomes (Hirayama and Iida, 2018; Underhill and Ozinsky, 2002a). The phagocytic processes involved cytoskeletal and actin rearrangement to force the particle internalisation (May and Machesky, 2001). Lysosome provides enzymes such as phospholipase (Akira *et al.*,