INTERACTION OF SILICA COLLOID AND IRON OXIDE NANOPARTICLES WITH MICE BONE MARROW DERIVED-DENDRITIC CELLS

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by

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TABLE OF CONTENTS

CHAPTER 2 : MATERIALS AND METHODS

CHAPTER 3 : RESULTS

CHAPTER 4 : DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACK	ammonium-chloride potassium
AEA	Animal ethics approval
Ag	Silver
APCs	antigen presenting cells
ARASC	Animal Research and Service Centre
BMDCs	bone marrow derived-dendritic cells
CCR7	chemokine (C-C Motif) receptor 7
$CD4^+$	helper T cells
$CD8^+$	cytotoxic T cells
CO_2	carbon dioxide
CoCr	cobalt-chromium
DCs	dendritic cells
DPX	Distyrene, a plasticizer, and xylene mounting medium
EtOH	Ethanol
FBS	fetal bovine serum
FDCs	follicular dendritic cells
Fe_3O_4	iron oxide
$FeCl_2.4H_2O$	ferric chloride
$FeCl_3.6H_2O$	ferrous chloride
FITC	Fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward scatter detector
GM-CSF	growth monocyte-colony stimulating factor

HLA-DR	human leukocyte antigen-antigen D related
HMDS	Hexamethyldisilazane
IFN-γ	interferon-γ
IL-4	interleukin 4
LPS	Lipopolysaccharide
MFI	mean fluorescence intensity
MHCI	major histocompatibility complex class 1
MHCII	major histocompatibility complex class 2
MLR	mixed leukocyte reaction
My D88	myeloid differentiation primary response gene 88
N_2	Nitrogen
NPs	nanoparticles
NH_4OH	Ammonia
NK	natural killer
NLRP3	nucleotide-binding oligomerization domain-like receptor protein 3
PBS	phosphate buffered saline
PDI	polydispersity index
RBC	red blood cells
ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute
SEM	scanning electron microscope
SiO_2	silica colloid
SPSS	Statistical Package for Social Sciences
SSC	side scatter detector
TCRs	T-cell receptors

TEOS	Tetraethylorthosilicate
Th1	type 1 T helper
Th2	type 2 T helper
TiO_2	titanium dioxide
TLRs	toll-like receptors
TLR4	toll-like receptor 4
Tregs	regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon β
ZnO	zinc oxide
ZP	zeta potential
γ-Fe_2O_3	ferric hydroxide
%	percent
ºC	degree Celcius
γ	Gamma

LIST OF APPENDICES

INTERAKSI NANOPARTIKEL BAHAN LEKAT SILIKA DAN BESI TEROKSIDA DENGAN SEL DENDRITIK DARIPADA SEL SUM-SUM TULANG TIKUS

ABSTRAK

Sel dendritik (SD) merupakan komponen penting imuniti semulajadi badan yang mana berfungsi untuk mengatur imuniti penyesuaian diri. SD bertindak sebagai kunci yang mengawal keseimbangan homeostasis imunisasi kita, sebagai sel pesembah antigen profesional, SD telah disasarkan dalam pelbagai aplikasi perubatan seperti di dalam penyelidikan vaksin dan pembangunan terapi imun berdasarkan kebolehan sel dendritik dalam memulakan imuniti semulajadi badan dan seterusnya mengaktifkan gerak balas imun perolehan. Kegunaan nanopartikel (NP) telah menunjukkan potensi yang hebat dalam mengaktifkan sel dendritik yang diperoleh daripada sum-sum tulang (SDST) mencit yang mengarah kepada kematangan SD, yang mana akan mendorong kepada tindakbalas imuniti semulajadi dan perolehan. Tujuan kajian ini adalah untuk menghasilkan dan mempercirikan dua jenis NP iaitu NP bahan lekat silika dan besi teroksida, untuk menentukan morfologi dan status keaktifan SDST apabila didedahkan kepada NP tersebut dan juga untuk memutuskan pengangkutan dan kemasukan kedua-dua NP ke dalam SDST. NP silika dan besi yang berbentuk sfera telahpun berjaya disintesis dan dicirikan dengan saiz hidrodinamik 174.7 nm ± 2.20 dan 214.0 nm ± 2.85, masing-masing. Kemudiannya, morfologi SD yang dihasilkan daripada sel sum-sum tulang mencit sebaik sahaja didedahkan kepada 25 ng/mL NP silika dan besi teroksida selama 24 jam telah

diperhati melalui mikroskop imbasan elektron, yang mana mikroskop elektron berkenaan telah menunjukkan pengambilan NP ke dalam membran sel oleh SDST. Status pengaktifan SD setelah terdedah kepada NP silika dan besi teroksida telah ditentukan melalui analisis flow sitometri, yang mana keputusannya mendapati ekspresi penanda permukaan sel yakni; MHCII, CD86 dan CD11c bertambah bagi SDST yang terdedah kepada NP silika dan besi teroksida berbanding SDST yang tidak terdedah kepada mana-mana NP. Tambahan pula, kemasukan intrasel NP ke dalam SDST telah disahkan dengan mikroskop laser yang mendapati NP telah disimpan di dalam membran sel, membuktikan pengambilan NP oleh SDST. Selain itu, kadar kepekatan rembesan pro-radang IL-12p70 di dalam supernatan SDST yang terdedah kepada NP silika dan besi teroksida adalah lebih tinggi berbanding SDST yang tidak terdedah kepada mana-mana NP. Sementara penemuan-penemuan ini mededahkan beberapa aspek yang menjanjikan NP silika dan besi teroksida sebagai calon bagi aplikasi terapi yang berkesan pada masa depan, penemuan ini masih memerlukan banyak lagi penyelidikan sebelum boleh diintegrasikan ke dalam pembangunan agen penghantar ubatan, vaksin dan terapi imun pada masa depan.

INTERACTION OF SILICA COLLOID AND IRON OXIDE NANOPARTICLES WITH MICE BONE MARROW DERIVED-DENDRITIC CELLS

ABSTRACT

Dendritic cells (DCs) are an important component of innate immunity, which modulate the adaptive immunity. DCs act as a key sentinel in maintaining our immune homeostasis and as professional antigen-presenting cells (APCs). DCs have been targeted in various medical applications such as in vaccine research and immunotherapeutic development due to the ability of activated DCs in initiating the innate immune response and consequently trigger the adaptive immune response. The use of nanoparticles (NPs) have shown a great potential in activating bone marrow derived-dendritic cells (BMDCs) that lead to DCs maturation, which in turn will induce the innate and adaptive immune responses. The aims of this study is to produce and characterise two types of NPs namely silica colloid NPs and iron oxide NPs, to determine the morphology and activation status of BMDCs upon exposure to NPs and also to determine the uptake and intracellular localisation of both NPs by BMDCs. The spherical silica colloid and iron oxide NPs were successfully synthesised and characterised with the hydrodynamic size of 174.7 nm ± 2.20 and 214.0 nm ± 2.85, respectively. Then, the morphology of generated DCs derived from the bone marrow stem cells of female BALB/c mice after 24 hours exposure to 25 ng/mL silica colloid and iron oxide NPs were observed through a scanning electron microscope imaging, whereby the electron microscopy showed that the BMDCs internalised the NPs into their enclosed membrane. The activation status of BMDCs

upon exposure to silica colloid and iron oxide NPs were determined through flow cytometry analyses, in which the expression of DCs surface markers; MHCII, CD86 and CD11c were enhanced in the BMDCs that were exposed to the silica colloid and iron oxide NPs as compared to the BMDCs without NPs exposure. Furthermore, the intracellular localisation of NPs into BMDCs was confirmed by confocal laser scanning microscope, where NPs were deposited inside the membrane of the cells indicating the uptake of NPs into the BMDCs. Besides, the concentration of pro-inflammatory cytokine IL-12p70 in the cell culture supernatant of BMDCs exposed to silica colloid and iron oxide NPs were higher than the BMDCs that do not exposed to NPs. Whilst these results revealed several promising aspect of the silica colloid and iron oxide NPs as a future candidate for an effective therapeutic applications, these findings warrant further research before they can be integrated into the development of future drug delivery agent, vaccine and immunotherapeutic treatments.

CHAPTER 1

INTRODUCTION

1.1 Human Immune System

Human immune system made up of diverse plethora of cells, tissues and molecules that guarding and protecting the body against various microbes, pathogens, bacteria or viruses invasion. The word ‘immunity’ refers to the universal ability of the host (human immune system) to resist or either destroys the predation of microbes (Hoebe *et al.*, 2004). The biggest dichotomy in immunity categorized into two general types of reactions which are an innate immunity and an adaptive immunity. These two general types of immunity are different in respect to the time taken for the immune response to occur, the period it takes to reacts against the pathogenic infection, the types of cells involved in each reaction and also the specific pathway mechanism for different classes of microbes. Despite the differences, both innate and adaptive immunity complement and rely on each other to maintain the balance of immune homeostasis.

1.1.1 Innate immunity

Innate immunity is essential to induce protection against various pathogenic infections and it has been known as the first line of defence of immune system (Uto *et al.*, 2009). The major components of the innate immunity are physical barriers (e.g. skin mucosal membrane of gastrointestinal tracts and respiratory tracts), phagocytic leukocytes (e.g. neutrophils, basophils, macrophages), antigen-presenting cells (APCs) such as dendritic cells (DCs), natural killer (NK) cells and plasma proteins. Innate immunity is a non-specific reaction that made up of those physical and chemical barriers that function to remove the pathogenic materials from the body or to limit the ability of the pathogen from spreading throughout the body. Besides, the innate immunity is activated immediately after recognizing the pathogen attacks and the release of their chemical properties. The innate immunity is important to induce the activation of consequent adaptive immunity.

1.1.2 Adaptive immunity

Other than the non-specific and natural innate immunity, adaptive immunity is known to be more specific and more potent in removing the invading pathogens (Uto *et al.*, 2009). The adaptive immunity also called as an acquired immunity, gets into action towards the pathogens invasion that is capable to pass through the first line of defence which is the innate immunity. Unlike the mechanism of innate immunity that destroy the pathogens based on the general information of the pathogen threats, the adaptive immunity is initiated by the exposure to the pathogens and its develop an immunological memory towards the threats and maintain the immune system

accordingly. The components of adaptive immunity comprises of the T lymphocytes and B lymphocytes that mediate a cell-mediated immunity and humoral immunity, respectively.

B lymphocytes are derived; formed and matured in the bone marrow which contribute to its name "B lymphocytes" and migrate into the lymphatic system to circulate throughout the body. The cells mature while encountering an antigen and express membrane-bound antibodies on the cell surface. The mature B lymphocytes differentiated into either memory B lymphocytes or effector B lymphocytes (plasma cell). Both cells secrete a highly specific protein molecules or antibodies that identify any free pathogens roaming around in the body and bind to the specific antigen that present on the surface of infected cells or freely float in the cell's body prior to the antigen destruction. The special ability of memory B lymphocytes assist the immune system to remember the disease and prevent re-infection in the future (Kurosaki *et al.*, 2015).

Another type of cellular components that made up the adaptive immunity is T lymphocytes. T lymphocytes are formed in the bone marrow, but it migrates to the thymus to mature and becoming T lymphocytes, thus the name. Instead of developing the expression of T-cell receptors (TCRs) to sense a specific protein sequence, T lymphocytes also express other receptors known as CD4 and CD8 receptors. TCRs only recognise antigens that are attached to a certain membrane-bound surface receptor on APCs, called as Major Histocompatibility Complex class 1 (MHCI) and class 2 (MHCII). The naïve T lymphocytes undergo maturation upon encountering with antigens by the up-regulation of MHCI and MHCII, co-

stimulatory molecules and adhesion molecules and differentiated into three types of mature T lymphocytes; CD4 helper T lymphocytes, CD8 cytotoxic T lymphocytes and regulatory T cells (Tregs). The CD4 activates B lymphocytes and attract APCs macrophages, the CD8 creates pores in the infected cells to trigger an apoptosis which in turn will eventually kill the infected host cells and Tregs reduce the possibility of autoimmune disease by helping the cells to distinguish between self and non-self molecule (Apostolou *et al.*, 2002).

As a whole, the response time of adaptive immunity is slower compared to the fast reaction of innate immunity but the specificity of adaptive immunity is highly specific and able to discriminate the differences between the pathogen and non-pathogen molecular structures rather than only specific for molecular patterns of general pathogens as in innate immunity. Regardless of the advantages and disadvantages of both types of immunity, a proper consideration should be made in maintaining the innate and adaptive immunity as they depend heavily on each other.

1.2 Dendritic Cells

1.2.1 Background and dendritic cells history

Steinman and Cohn were the first who discovered DCs almost four decades ago. They found a group of cells in the spleen with unusual shapes and movements that differed from macrophages, with a tree-like stellate or 'dendrites' (Steinman and Cohn, 1973). Figure 1.1 shows cytoplasmic veils and dendrites protruding from the main body of DCs, which is prominent especially after antigen stimulation. DCs were later found to exist in lymphoid and non-lymphoid tissues.

The successful generation of large numbers of DCs from $CD34^+$ bone marrow precursor cells in early 1990s (Inaba *et al.*, 1992a) and later from $CD14^+$ monocytes (Silveira *et al.*, 2013) allowed more studies on DC properties and functions to be carried out. These include the interactions of DCs with other immune cells and the efficacy of DC-based vaccines in pre-clinical and clinical aspects (Wieder, 2003) including cancer immunotherapy (Alamino *et al.*, 2016; Dudek *et al.*, 2013; Liu *et al.*, 2015; Sánchez-Paulete *et al.*, 2016; Sennikov *et al.*, 2015), organ transplantation (Thomson *et al.*, 2016) autoimmune diseases (Tian *et al.*, 2016) and allergic diseases (Froidure *et al.*, 2015).

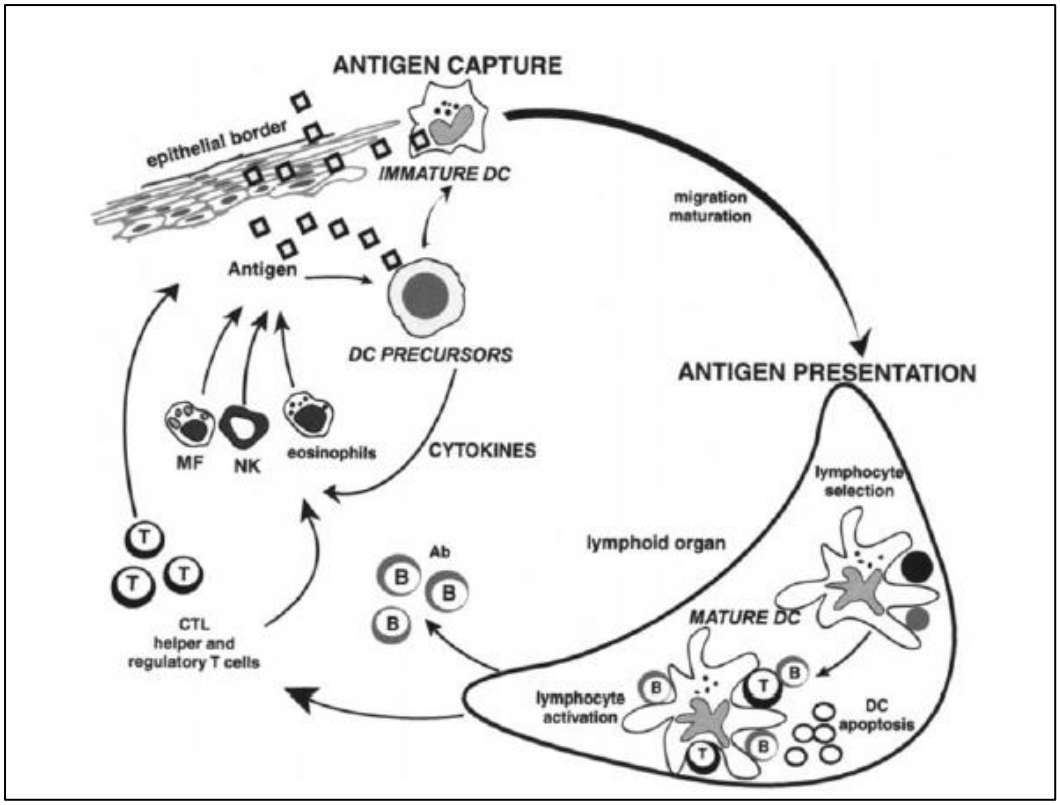

Figure 1.1. The life cycle of DCs. Circulating immature DCs directly encounter pathogens and induce cytokines secretion, which in turn can activate other leukocytes e.g. eosinophils, NK cells and macrophages. After antigen capture, the immature DCs migrate to lymphoid organ and present the MHC-peptide to activate T lymphocytes. The activated T lymphocytes help terminal maturation of DCs that allows lymphocytes proliferation and differentiation. It will migrate and reach the injured tissue and produce a more potent immunological response. Hence, DCs are critical for the induction of immunological tolerance, as well as the regulation of T-cell mediated immune response (Banchereau *et al.*, 2000).

1.2.2 Function and importance of dendritic cells

To date, DCs have been proved to be one of the most essential cells that can initiate and modulate the immune response. The functions of DCs can be divided into three main categories: (1) antigen presentation and activation of T lymphocytes, (2) maintenance of immune tolerance and (3) maintenance of immunological memory of B lymphocytes, conducted by follicular DCs.

Among these, the most prominent roles for DCs are the processing and presentation of antigens for the differentiation and activation of T lymphocytes. Immature DCs circulate throughout the peripheral lymphoid tissues to sample and capture foreign pathogens. The captured pathogens are internalized into specialized organelles, phagosomes and endosomes. When both compartments mature, they undergo molecular changes, for instance, fusion with other organelles that carry degrading enzymes as lysosomes. Then, antigenic peptides in the late lysosomes or endosomes can be generated and loaded onto MHCII molecules. The molecular complex is then transported to the cell surface for antigen presentation to T lymphocytes (Burgdorf and Kurts, 2008). The immature DCs become mature DCs during the process of capturing, processing and presenting the antigens (Lutz and Schuler, 2002). An adequate antigen presentation signal by DCs to T lymphocytes induces cytokine secretion and therefore activates effector T lymphocytes to get into action.

After the thymic selection, the T lymphocytes undergo differentiation and divide into either effector T lymphocytes [e.g. helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$)], and Tregs. A recent study by Alamino *et al*. (2016) highlighted the importance of

enhancing the ability of DCs to stimulate the effector T lymphocytes, particularly cytotoxic T lymphocytes that have potential for DC-based immunotherapy (Alamino *et al.*, 2016). Effector T lymphocytes and Tregs are part of cell-mediated immunity of adaptive immune response and play their own roles in the maintenance of the immune tolerance and protect the body against microbes.

Besides activating immune response, DCs also play an important role in maintaining immunological tolerance (Wieder, 2003), which occurs in the thymus (central tolerance) and in the peripheral lymphoid organs (peripheral tolerance). In the thymic medulla, DCs present self-antigens in the context of MHC molecules and thymocytes with high affinity receptor to self-antigens are eliminated via negative selection, immunity elimination against 'self' due to their high affinity to self-antigens. In the thymic cortex, macrophages digest the dying thymocytes which fail to go through positive selection. T lymphocytes should be tolerant to self-antigens prior to T lymphocyte activation in order to avoid the reactivation of auto-reactive T lymphocytes (Banchereau and Steinman, 1998). In addition, DCs also maintain peripheral tolerance through cytokines or cell-contact dependent mechanisms to prevent the induction of immune response on self-reactive T lymphocytes that escape the central tolerance (Mahnke *et al.*, 2002). Therefore, immunological tolerance mediated by DCs is essential in protecting our immune system from autoimmune diseases since DCs might promote tolerance through generation and maintenance of Tregs as well as by the induction of T lymphocytes unresponsiveness (Ganguly *et al.*, 2013).

Follicular dendritic cells (FDCs) are identified based on their specific morphology and the ability to capture immune-complexed antigens in B lymphocyte follicles in the germinal centers. FDCs directly sustain the viability, growth and differentiation of B lymphocytes (Banchereau and Steinman, 1998). FDCs recognise antigens by toll-like receptors (TLRs) and help in skewed B lymphocytes response and subsequently boosting adaptive immune responses (Aguzzi *et al.*, 2014). Altogether, DCs are crucial in maintaining the immune homeostasis, directing T and B lymphocytes on one hand while upholding immunological tolerance on the other.

1.2.3 Maturation of dendritic cells

As introduced earlier, DCs originated from the hematopoietic stem cells in the bone marrow and they are distributed within all lymphoid tissues as immature DCs. Immature DCs serve as a primary lineage of immune protection as they are recruited to the inflammatory sites in peripheral tissues after pathogen invasion. At this stage, the immature DCs internalize the pathogens via endocytosis pathways such as phagocytosis, pinocytosis, macropinocytosis (Kou *et al.*, 2013) and receptor-mediated endocytosis including caveolae- and clathrin-dependent and independent pathways (Pelkmans and Helenius, 2002). Immature DCs show high efficiency in the antigen uptake and processing which induce an incredible maturation process (Uto *et al.*, 2009).

The distinction between immature DCs and fully mature DCs are described based on phenotypic and functional changes (Dudek *et al.*, 2013). While the immature DCs are becoming mature DCs, they undergo various changes that in turn, culminate in

the complete transition from antigen capturing cells to APCs. The DCs maturation process involves a redistribution of MHC molecules to the surface of DCs, morphological changes, increase in the surface expression of co-stimulatory and adhesion molecules, cytokines and proteases. The mature DCs that have undergone these dramatic changes have lost the ability to capture antigen, but they obtain another capacity, e.g. the presentation of antigen to T lymphocytes for the subsequent activation of T lymphocytes.

Co-stimulatory molecules such as CD80, CD83, CD86, HLA-DR, IL-12 and CD40 are upregulated in mature DCs, while the cytokine production of IL-10 is reduced (Daneshmandi *et al.*, 2015; Liu *et al.*, 2015). Large amounts of cytokines are produced by mature DCs that promote the recruitment of monocytes and upregulation of Chemokine (C-C Motif) Receptor 7 (CCR7). CCR7 is a chemokine receptor required for the migration of DCs into lymphatic vessels and their localisation to the T lymphocyte areas (Lanzavecchia and Sallusto, 2001). DCs then undergo the maturation process by upregulating T lymphocyte co-stimulatory molecules such as CD40, CD80, CD83 and CD86 (Cybulsky *et al.*, 2016). T lymphocytes will bind to MHC antigen complexes and differentiate into $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, Tregs and memory T cells. In response to multiple pathogens, T lymphocyte activation and differentiation lead to appropriate generation of responses either in the form of tolerance, memory and cytotoxicity in order to maintain the immunological homeostasis.

1.2.4 Uptake capacity of dendritic cells

The initiation of immune responses can be described from the interaction of three immune parties that focused on antigens, lymphocytes and APCs which is, in this context are referring to DCs. DCs serve as the modulator and acts as a key sentinel in bridging both innate and adaptive immunity, where DCs capture and pass on the cellular information from the external environment to the adaptive immune cells (Banchereau *et al.*, 2000; Banchereau and Steinman, 1998). DCs are important to induce the immunological tolerance as well as to regulate the T-lymphocytes mediated immune response (Banchereau *et al.*, 2000). The uptake of intracellular or extracellular antigen by DCs is mediated by DCs uptake mechanisms, for example phagocytosis, macropinocytosis and receptor-mediated endocytosis (Figure 1.2). This antigen acquisition processes lead to presentation of the processed antigen peptides onto MHCI, MHCII and up-regulating the co-stimulatory molecules such as CD40, CD80 and CD86 to initiate the interaction with T lymphocytes of the adaptive immune system that consists of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells phenotypes (Vallhov *et al.*, 2007).

Today's advancement in medicine has provided an alternative to combat the drawbacks on the use of traditional drugs, have emerged plentifully by means of use of NPs that would improve the organ-targeting capacity especially in cancer patient, delivery of macro-molecules and minimizing the side effects of the traditional drugs (Moghimi *et al.*, 2005). The NPs internalized by the APCs via endocytosis pathway that classified based on the type of proteins that play role in the endocytosis process.

One of the most important distinct mechanisms of antigen acquisition by the family of APCs which includes macrophages, neutrophils and DCs is phagocytosis process. Neutrophils rely on their response against invading microbes mechanism called as 'oxidative burst', while macrophages depends on their lysosomal proteolytic activity to eliminate the phagocytosed pathogens which is critical in maintaining the safety environment in innate immune system. In comparison to both macrophages and neutrophils, DCs does not merely eliminate the pathogens but they also preserve any useful information from the internalized particles that will contribute their function in activating T lymphocytes and initiating the adaptive immune response.

The cellular mechanism for phagocytic pathway for DCs starts with the recognition and uptake of pathogens, apoptotic cells or infected cells in the peripheral tissue and DCs process the antigens from these pathogenic cells into peptides form and load the peptides fragments on MHC class I or class II molecules. DCs then migrate to the secondary lymphoid organs to present the antigens to T lymphocytes recognition and activation that will initiate the adaptive immune response (Savina and Amigorena, 2007). Phagocytosis process are mediated by several types of receptors such as Fc receptors, CD36 scavenger receptor, C-type lectins and complement receptors that recognise the ligands present on the pathogens and internalize them (Roche and Furuta, 2015).

The DCs with high phagocytic activity usually performed by immature DCs that will develop into mature DCs upon encountering proinflammatory signals or TLRs ligands and subsequently loss their phagocytic activity due to the changes activity in Rac and Cdc42 (Nobes and Marsh, 2000).

Another endocytosis mechanism involved in the antigen acquisition of DCs is macropinocytosis, a non-specific uptake of solute or soluble molecules, nutrients and also antigens and often known as "cell-drinking" (Liu and Roche, 2015). Macropinocytosis is an actin-dependent formation that rise from the ruffles of plasma membrane forming a large vesicles called as macropinosomes that has a different forms compared to other endocytic vesicles (Liu and Roche, 2015; Norbury, 2006) with diameter size range of 0.2 up to 5 µm (Hewlett *et al.*, 1994; Swanson and Watts, 1995). Macropinocytosis mediates the non-specific soluble antigen uptake into cellular compartment via the endocytic vesicles macropinosomes. The antigens that are enclosed within the macropinosomes are transferred into endosomal compartment that fuse with lysosomal compartment in order to process the antigen. After the antigen processing activity took place, the macropinocytosed antigens undergo degradation step and being loaded onto MHCII molecules (Liu and Roche, 2015). Since immature DCs can perform a great macropinocytosis activity, (Sallusto *et al.*, 1995) found that the human monocyte derived-dendritic cells (MDDCs) cultured with GM-CSF and IL-4 can uptake the fluid-phase antigen for about 1 – 1.5 Pl and over 40 % of the cell volume per hour (Sallusto *et al.*, 1995). In some extent, the mechanism of macropinocytosis and phagocytosis are quite similar in which upon stimulation with external stimuli that induce the maturation of DCs, the macropinocytosis process will be terminated and the process is irreversible.

In addition to the non-specific antigen acquisition such as macropinocytosis, DCs also have several receptors on their cell surface that facilitates the uptake of antigen into the cellular processing compartment via a clathrin-mediated endocytosis

pathway. This receptor-mediated endocytosis bind the small soluble molecules antigen to a various types of receptors present on DCs such as Fc receptors, complement receptors and C-type lectins that will be internalized by either clathrin-coated or non-clathrin-coated vesicles. Antigen captured in the clathrin-coated vesicles are send to endosomal compartment and to the antigen processing vesicle compartments for proteolytic enzyme degradation and for the formation of peptide loaded onto MHCII as to activate the resting T lymphocytes and to initiate the specific adaptive immune responses (Liu and Roche, 2015; Roche and Furuta, 2015).

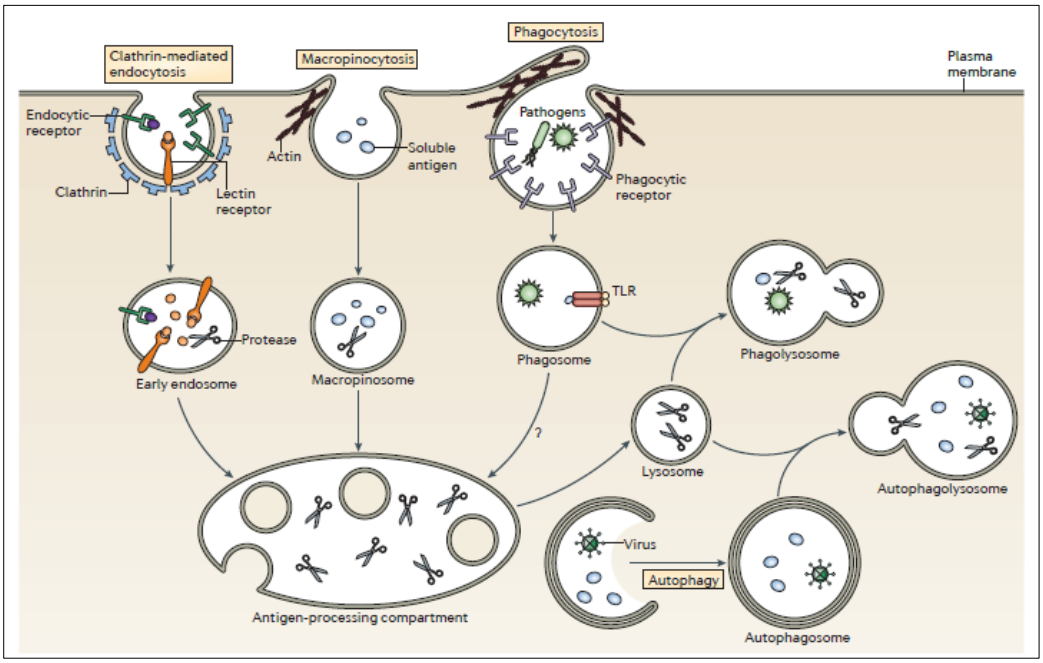

Figure 1.1. Endocytosis of antigens in APCs that involve phagocytosis, macropinocytosis and clathrin-mediated endocytosis pathway (Roche and Furuta, 2015).

1.2.5 Bone marrow derived-dendritic cells

Recently, DCs have been widely used as target cells for the production or induction of immunomodulatory and anti-inflammatory effects. Therefore, DCs are routinely cultured *in vitro* to assess their functions and actions (Inaba *et al.*, 1990; Romani *et al.*, 1989) and to determine basic pathophysiologic mechanism, to generate DC-based immunotherapy (Dudek *et al.*, 2013) and to study their adjuvant properties (Romani *et al.*, 1989; Steinman, 1991).

The basic procedure to obtain bone marrow from femur of mice and to cultivate healthy bone marrow derived-dendritic cells (BMDCs) involves complex and multistep procedures (Madaan *et al.*, 2014). BMDCs from mice and rats are cultured *in vitro* by using Growth Monocyte-Colony Stimulating Factor (GM-CSF) (Bowers and Berkowitz, 1986; Inaba *et al.*, 1992a; Talmor *et al.*, 1998). Lutz and colleagues have produced a 10-day procedure for generating BMDCs and have been widely used by researchers to generate DCs until now (Lutz *et al.*, 1999).

1.3 Nanoparticles

1.3.1 Definition and uses of nanoparticles

In today modern world, nanoscience and nanotechnology have been regarded the most important research in modern science and nanotechnology and it allow multi-disciplinary professionals including scientist, engineers, chemists and physicians to joint venture and investigate the molecular and cellular levels that will lead to the advancement in the life sciences and healthcare field. The major advantages offer by nanoparticles (NPs) are due to their unique size and great physicochemical properties (Akbarzadeh *et al.*, 2012). In the recent years, the advancement in nanoparticle research has impacted and benefited multiple industries by taking advantage of their special qualities of small "atomic-sized". The unique properties may affect the immune system and human health in such a way that differs from those seen with larger particulates or gases (Chang, 2010).

NPs share a quite similar size range as ultrafine particles, however the distinction between these two lies in their origin. The ultrafine particles refer the particles that are produced and released as an emissions result from industrial byproducts or everyday life, whereas NPs are engineered for specific purposes. NPs defined as particles with a diameter size of less than 100 nm (Hardy *et al.*, 2012; Hardy *et al.*, 2013) and the particles can be classified into three categories; naturally occurring NPs (e.g. volcanic eruptions or viral infection), anthropogenic NPs which produced inadvertently from human activity (e.g. combustion from vehicles engine, power

plants byproducts and incinerators) and engineered NPs produced for various industrial products (Chang, 2010; Mohamud *et al.*, 2013).

NPs have been utilized in various disciplines such as cosmetics, pharmaceutical application (Coester *et al.*, 2006), DNA fingerprinting system (Choi *et al.*, 2008), involve in the development of drug deliveries (Bender *et al.*, 1996; Broos *et al.*, 2010; Chan *et al.*, 2010; des Rieux *et al.*, 2006) and diagnostics to detect biomarkers of heart disease, cancer theranostics (Gobbo *et al.*, 2015; Hoang *et al.*, 2015) and infectious agents (Akbarzadeh *et al.*, 2012; Verma *et al.*, 2013).

1.3.2 Synthesis of nanoparticles

The experimental silica colloid (SiO_2) NPs and iron oxide (Fe_3O_4) NPs used in this study were synthesised via the so-called 'Stöber process' and 'co-precipitation' methods. Stöber process is regarded as the simplest and most effective technique to prepare monodisperse silica particles since the reactants used are normal and the reaction process is controllable, low-cost and easy to be carried out (Wang *et al.*, 2010). The preparation of monodisperse SiO_2 NPs generally started with the hydrolysis and condensation of alkoxysilanes or tetraethylorthosilicate (TEOS) in a mixture of alcohol, water and ammonia which act as a catalyst for the whole reaction. This ammonia-catalyzed reaction of TEOS with water in low molecular weight of alcohols is famous for the production of monodisperse silica particles in spherical morphology (Nozawa *et al.*, 2005) with size range of 0.05 to 2 µm (Stöber *et al.*, 1968).

Generally, the hydrolysis reaction gives the hydrolysed TEOS monomer [(OR)3Si(OH)]

$$Si(OR)_4 + H_2O \rightarrow (OR)_3Si(OH) + ROH$$

The intermediate reaction product then condenses to form silica particles according to the below chemical equation

$$(OR)_3Si(OH) + H_2O \rightarrow SiO_2 + 3ROH$$

The chemical reaction is a simplification of the complex condensation process that leads to the formation of silica particles.

On the other hand, there are various methods that can be adopted to prepare the Fe_3O_4 NPs incuding microemulsion, thermal decomposition and co-precipitation method (Mahdavi *et al.*, 2013). However, the co-precipitation method was used in this study because of its low cost and convenient steps despite of its capability in producing fine, high-purity and stoichiometric particles (Chen *et al.*, 2005). As its reaction name implied, the co-precipitation is conducted by simultaneous precipitation of ferrous (Fe^{2+}) and ferric (Fe^{3+}) salt mixtures with the addition of strong base, in this case NaOH was used. The Fe $(OH)_2$ and $Fe(OH)_3$ were formed by the hydroxylation of the ferrous and ferric ions under anaerobic conditions (Jolivet *et al.*, 2004). After that, the formation of Fe_3O_4 NPs can be obtained by collecting the black precipitate via magnetic separation and the chemical equation for this reaction is as follows:

$$Fe^{3+} + 3OH^- \rightarrow Fe(OH)_3$$

$$Fe(OH)_3 \rightarrow FeOOH + H_2O$$

$$Fe^{2+} + 2OH^- \rightarrow Fe(OH)_2$$

$$2FeOOH + Fe(OH)_2 \rightarrow Fe_3O_4 \downarrow + 2H_2O$$

The reaction occurs very fast and magnetite crystals can be seen as soon as the strong base is added into the salts mixture. The reaction should be oxygen free in order to prevent the oxidation of magnetite NPs (Fe_3O_4) to hematite NPs; ferric hydroxide (γ-Fe_2O_3) (Mahdavi *et al.*, 2013).

1.3.3 Advantages of nanoparticles in biomedicine

The unique physicochemical properties of NPs have offer great opportunities for collaborations with biomedical research that involves particularly specialized living organisms and require thorough investigations by both material scientist and biomedical engineers. Despite many challenges that have to be faced when dealing with the NPs, a well-established concepts, theories and methods need to be comprehend properly to translate the exclusive properties of NPs into a useful and creative biomedical applications.

The benefits of this new research avenue to human health and civilization is indeed immeasurable (Chang, 2010) and in the recent decades, there is a remarkable increased in the use of NPs in medical applications, and they are foreseen to be widely applied in medical uses. Recently, various types of NPs are used for biological practices, for example, silica NPs have been used in diagnosis and bioanalysis (Tian *et al.*, 2007), cell imaging (Tsai *et al.*, 2008) and gene or drug delivery (Lu *et al.*, 2007; Slowing *et al.*, 2007), gold NPs involve in the theranostics and plasmonic phototermal therapy (PPT), magnetic metal NPs (e.g. Fe_3O_4 NPs) being used for magnetic resonance imaging (MRI) and hyperthermia therapy, quantum dots offer visibility alternative to positron emission tomography (PET)

imaging, plasmonic NPs enhance the surface plasmon and facilitate the naked eye readout (Giner-Casares *et al.,* 2016) and organic NPs conjugated with DNA, protein or drug and acts as adjuvant in drug delivery activity.

The uniqueness of NPs properties related with the nano-meter size particles that often range between 1 up to 100 nm and possess different physical and chemical composition compared to their bulk counterparts (Moghimi *et al.,* 2005). The diminutive size of NPs facilitates their interaction or uptake by targeted cells, as well as enabling them to interfere with specific subcellular components which lead to a less toxic and more efficient therapeutic activity (Mohamud *et al.,* 2013). By virtue of their tiny size and upon surface functionalisation with appropriate ligands and synthetic polymers, NPs can serve as drug carrier that target the specific cells and area inside human body after being injected intravenously or subcutaneously (Allen and Cullis, 2004; Krämer *et al.*, 2004; Moghimi *et al.*, 2001). This *in vivo* approach will eventually enhance the detection sensitivity in medical imaging such as MRI, improve the efficiency of therapeutic activity and nevertheless, can also suppress the side effects caused by conventional drugs or treatments.

The interaction between both NPs and biomedicine is making their way in the fast-pace activity in the research and development area of interest, however it is also pertinent to consider the issues that associated with the so-called nanotoxicity and thorough understanding on the physical and chemical properties of each types of NPs on each cells types. For example, studies found that the local or systemic toxicity which associated with exposure to metal NPs would impact acute and chronic nano-bio interactions at the locations where the NPs are being deposited (Lee *et al.,* 2016;

Shao *et al.*, 2014). Hence, the consideration on the nanotoxicity effect of NPs to the cells is required to ensure a proper therapeutic alternative is made while addressing the safety of the nanoparticulate systems to be consumed by human immune system.

1.3.4 Silica colloid and iron oxide nanoparticles

The emergence of NPs role in biomedical application including the NPs-mediated targeted drug delivery system have caused a great effect that lead to the development of new therapeutic and diagnostic tools (Liu *et al.*, 2007). The research on this aspect has expanded tremendously in the recent years and there are various products available in the market, for example natural NPs like liposomes, dendrimers, and microbubbles and engineered nanovehicles such as silica NPs (Liu *et al.*, 2006; Moghimi *et al.*, 2005) and magnetic NPs (Chertok *et al.*, 2010). Nanomaterials are capable to interact with biological system at molecular level with high specificity because of what we called as nano-effect (Jiang and Papoutsakis, 2013; Silva, 2006).

Silica is a versatile natural material component made up from sand and glass element that has been employed in engineering and material sciences for years. It has received spotlight for biomedical purposes due to its properties of being relatively benign material with no toxicity and good biocompatibility (Liu *et al.*, 2007). The nano-effect can be successfully done with a proper understanding on the NPs size, surface properties and physicochemical properties. The silica NPs possess the unique features including tunable sizes and morphology, uniformly arranged particles, high surface area-to-volume ratio (Sahoo *et al.*, 2014), high chemical stability than other

polymer-based NPs, the functional inner and outer surface of the particle which allow selective functionalisation, unique porous structure that is required to prevent premature release in drug delivery system and good *in vivo* biocompatibility (Asefa and Tao, 2012; Giret *et al.*, 2015) and hemocompatibility (Slowing *et al.*, 2009).The advantages of silica NPs were not only in industrial applications (e.g. acts as catalysts, pigments, fillers) but also in the scientific field that involve medicinal practice, for example, as a delivery system for cancer therapy (Lu *et al.*, 2007), as a multifunctional probe for cell imaging (Tsai *et al.*, 2008) and encapsulated magnetic NPs to be used in the diagnosis and therapy, as well as cell imaging (Chen *et al.*, 2010; Deng *et al.*, 2008).

Other than silica particles, hybrid nanostructures have also gained interest in the nanomedicine field because of their diverse physicochemical properties (Narayanan *et al.*, 2011). Magnetic NPs belong to the group of hybrid nanostructures that have been explored for variety of biomedical applications due to their unique mesoscopic chemical, physical, mechanical and thermal properties (Mahdavi *et al.*, 2013). Fe_3O_4 NPs is one of the magnetic NPs which happen to be non-toxic, biocompatible (with special surface coating) and can be purposely modified with drugs, proteins, antibodies, nucleotides or enzymes to adjust the surface chemistry of the NPs for better biological performance. Based on the uniqueness of magnetic NPs, they have been widely studied and applied as MRI contrast agents (Lee *et al.*, 2010; Mahdavi *et al.*, 2013; Narayanan *et al.*, 2011), induction of local hyperthermia towards an alternating magnetic field for cancer cells removal (Bae *et al.*, 2012; Mahdavi *et al.*, 2013), and for magnetically targeted drug delivery system (Talelli *et al.*, 2009).

1.3.5 Cellular uptake of nanoparticles

Recently, the use of engineered NPs in various technological applications have undergo tremendous amount of investigations. Regardless of many studies that have been conducted to determine the toxicity of certain types of NPs on cells, however there is very little information on the uptake mechanism of NPs (Kettler *et al.*, 2014). The knowledge of cellular uptake of NPs is crucial to evaluate the possible risk assessment of the NPs, to determine the functional behaviour of NPs according to their unique physicochemical properties and to improve the beneficial effects of NPs in biological applications (Kettler *et al.*, 2014; Patil *et al.*, 2007).

The diminutive size of NPs ease the penetration of NPs into the cellular barriers and understanding on the uptake of mechanism of NPs into cells should be kept in mind. A cell membrane of cell is a physical barrier to separate the external environment and the internal cellular compartment of cells and particles with sizes of 10 nm to 30 nm can easily cross the membrane (Lead and Smith, 2009). Hence, an endocytosis mechanism is a general type of uptake mechanism that works in overcoming this cellular barrier in its own specialized way. Endocytosis refers to the uptake of particulate substance (e.g. proteins) into the cells through the enclosure of cell membrane. Other than endocytosis, another uptake mechanism of NPs called as phagocytosis and pinocytosis are the internalization of particulate substances and small solute and the internalization of large amounts of solute, respectively (Mercer and Helenius, 2009). The pinocytosis mechanism can be divided into macropinocytosis (Kannan *et al.*, 2004) and receptor-mediated endocytosis (Chithrani and Chan, 2007).

Apart from the specific uptake mechanism that can be demonstrated by the NPs to get into the cellular compartment, the surface chemistry of the NPs itself can ascertain the protein corona and contribute to a significant impact on the biological performance of the NPs internalization by the cells (Lundqvist *et al.*, 2008; Patil *et al.*, 2007). The properties of NPs that influence their uptake by cells are the size of NPs (Foged *et al.*, 2005; He *et al.*, 2010; Rejman *et al.*, 2004; Win and Feng, 2005), surface charge of NPs (Foged *et al.*, 2005; He *et al.*, 2010; Patil *et al.*, 2007), the functional group of NPs (Alexis *et al.*, 2008) and the hydropholicity of NPs (Clift *et al.*, 2008).

The consideration and understanding on the uptake mechanism of NPs should be properly comprehends while constructing the nanomaterials as to ensure the functionality and biocompatibility of NPs with the cells go hand in hand.