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Immuno-regulatory Effects of Chitosan and Its Derivative on Dendritic Cells Maturation

Graduate School of Chosun University

Department of Marine Life Science

Phuong Hong Nguyen

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수지상세포 성숙화과정에 대한 키토산 및 그 유도체의

면역조절 효능 연구

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지도교수 정 원 교

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Graduate School of Chosun University

Department of Marine Life Science

Phuong Hong Nguyen

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위원장 조선대학교 교수 조태오 (인)

위 원 조선대학교 교수 정원교 (인)

위 원 조선대학교 교수 이준식 (인)

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응 구 엔 흥 프 헹
지도교수: 정원교
해양생명과학과
조선대학교 대학원

본 연구에서는 대식세포와 함께 인체 면역방어 시스템을 수성하는 중요인자인 수지상세포의 성숙화과정에 대한 키토산 및 그 유도체의 효능을 확인하였다. 해양 갑각류 유래 다당체인 키토산에 대한 대식세포 및 b/T 세포 관련 면역활성의 많은 효능 및 기전연구가 발표되었지만, 수지상 세포 분화에 따른 면역조절 활성은 보고된 바 없으며, 아울러 합성을 통해 얻어진 키토산 유도체에 대한 면역 약리학적 효능은 원천기술 및 소재로써, 그리고 학문적 연구자료로써 가치가 높을 것으로 사료된다. 따라서 본 연구는 동물 골수조직으로부터 분리한 monocyte 를 면역조절활성에 중요한 역할을 하는 수지상세포로 분화시켜 이들의 성숙화 과정에 키토산 및 그 유도체의 영향을 파악하였으며, 그 결과 NF-κB 및 MAPKs 기전을 통한 면역활성 조절을 통해 이들 해양바이오소재가 가지는 면역 조절 효능을 확인할 수 있었다.

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ABSTRACT

Immuno-regulatory Effects of Chitosan and Its Derivative on Dendritic Cells Maturation

Phuong Hong Nguyen
Advisor: Prof. Won-Kyo Jung, Ph.D.
Department of Marine Life Science,
Graduate School of Chosun University

Chitosan and its products (microparticles, nanoparticles, and its mixture with ovalbumin) have been reported to have immune-stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cells. They can regulate DCs maturation and development through the modulation of the surface markers such as class II, CD80 and CD86 and chitosan-stimulated DCs-released cytokines such as IL-12, TNF-α, and IFN-γ. Therefore, the use of chitosan and its derivatives used as immunological adjuvants to regulate both humoral and cell-mediated immunity is promising. Furthermore, different chitosan derivatives have showed different activities as well as their effects in immune system. In this study, we could investigate the regulation of chitosan (CS) and its derivative named chitosan-catechin conjugate (CSC) on murine monocyte-derived DCs maturation.

KEYWORDS: *chitosan derivatives, catechin chitosan, dendritic cells (DCs), DCs maturation*

I. INTRODUCTION

A. Introduction of chitosan and their derivatives

1. Structure and processing of chitosan

Chitosan known as a natural nontoxic biopolymer of the deacetylation of chitin is a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Chitosan is originated from the structural element in shell or the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. It is between 3800 to 20,000 Daltons for using the molecular weight of commercial chitosan on average. The deacetylation of chitin using sodium hydroxide as a reagent and water as a solvent is a common method for the synthesis of chitosan (Figure 1).

Chitin $(C_8H_{13}O_5N)_n$ shows the properties of a naturally abundant mucopolysaccharide. Chitin, supporting complement of crustaceans, insects, etc., is known to consist of 2-acetamido-2-deoxy-D-glucose through a β (1 → 4) linkage (Kumar, 2000) (Figure 1). From crab or shrimp shells and fungal mycelia we can obtain chitin. In the first case, it is associated between chitin production and food industries such as shrimp canning. In the second case, the production of chitosan-glucan complexes is involved with fermentation processes. Chitin has the natural functions as a structural polysaccharide, but differs from cellulose in its properties. Chitin has a highly hydrophobic and insoluble property in water and most organic solvents. Additionally, their high percentage of nitrogen compared with synthetically substituted cellulose can lead chitin and chitosan become commercial interest. These properties exert chitin become a useful chelating agent (Muzzarelli, 1973). Chitin and chitosan are reported as the functional materials because these natural polymers possess excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties, etc (Kumar, 2000). The processing of chitin and chitosan from crustacean shells mainly associate the degradation of proteins and the dissolution of calcium carbonate in crab shells. For example, the chitin is deacetylated in 40% sodium hydroxide at 120^{o}C for 1–3 h (Scheme. 1). This method produces 70% deacetylated chitosan. However, the high molecular weight and viscosity of chitosan may limit its use in several biological applications. For some specific applications, its degradation products, low-molecular weight chitosan (LMWC), chitooligosaccharides (COS), and monomers, were investigated to be much more functional.

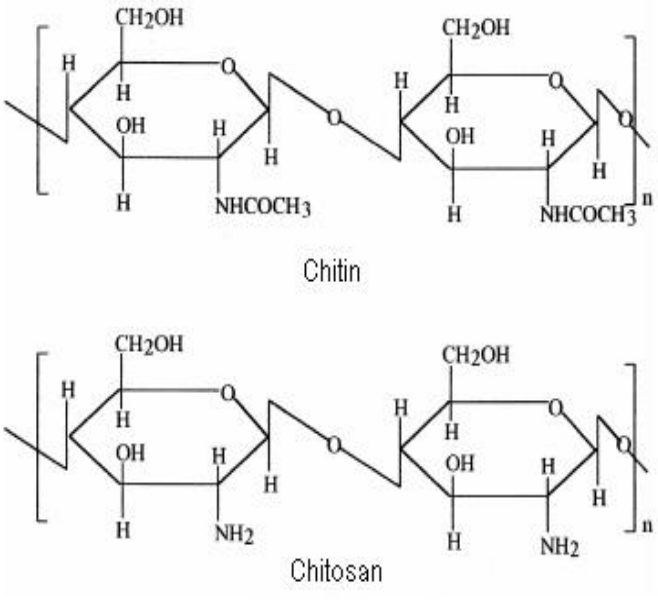
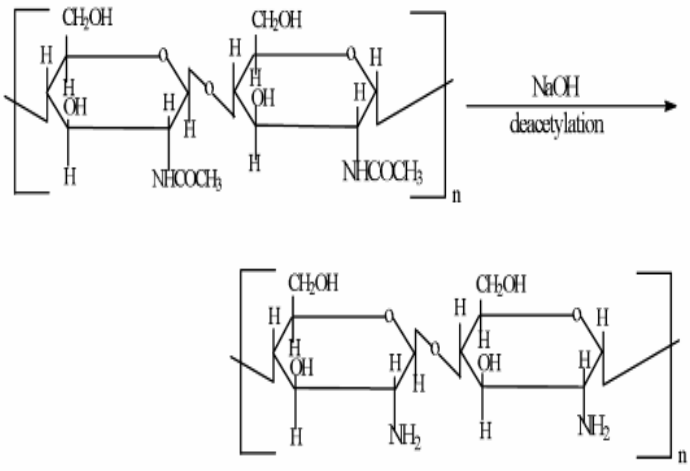


Figure 1. Structures of chitin and chitosan



Scheme 1. Processing of chitin and chitosan

Many methods of degradation such as chemical, physical and enzymatic method is being used to produce these degradation products (Figure 2). Both chitin and chitosan oligomers have additionally functional properties of bioactive effects. Additionally, low molecular weights and

short chain lengths have the properties of lower viscosity and solubility in neutral aqueous medium. In in vivo study, they have a property of absorption (Prashanth et al 2007). There are two methods to depolymerize chitosan and chitin. Firstly, acid degradative methods are not specific, the hydrolysis can randomly generate a large amount of monomers, and later the destruction of acid poses problems and also it is not economical (Kumar et al, 2004). Chemical method using strong acids is a very common and fast method to produce a series of chitooligomers, but this method might show some disadvantages such as high cost, low yield and residual acidity. The second is the enzymatic methods. Many degradation products of chitosan may be generated by a variety of methods. However, the enzymatic methods are getting importance because they permit regioselective depolymerization under mild conditions (Prashanth et al 2007). Additionally, the unwanted level of pyrogenicity caused by the presence of protein admixtures in such preparations cannot be calculated. The method of the enzymatic degradation of chitosan using chitinase, chitosanase, glucanase, lipase and some proteases can produce potent LMWC with high water solubility (Kumar et al, 2004).

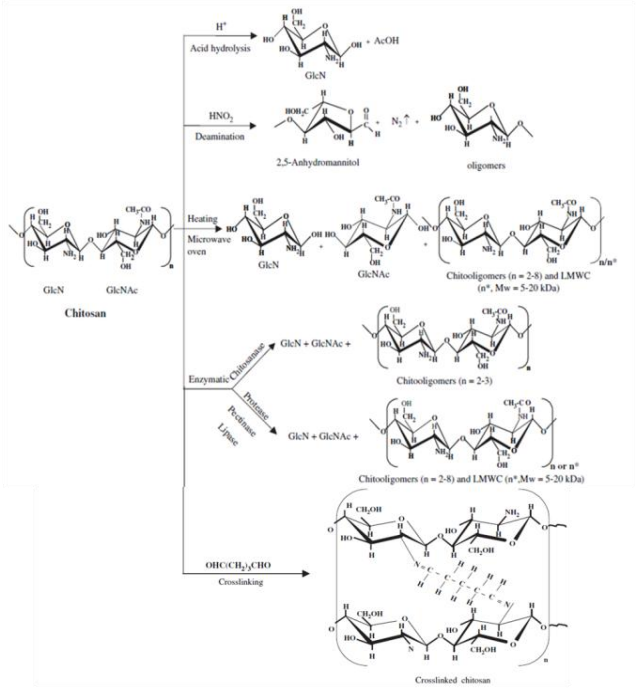


Figure 2. Depolymerization products and crosslinking of chitosan

2. Potential industrial and biomedical significance

In agriculture and horticulture, chitosan possess a rich history of being investigated for applications, and recently in biomedical investigation (Figure 3 and 4). Chitosan is reported their effect for natural seed treatment and plant growth enhancer, and as an ecological and friendly biopesticide agent that enhance the innate ability of plants to prevent themselves against fungal infections (Linden et al, 2000). Chitosan might also be applied in water processing engineering, filtration process, and potential industrial use (Ghosh and Urban, 2009). In biomedical uses, structural properties of chitosan showed their effect to rapidly clot blood, and have recently displayed approval in the United States and Europe for applications in medical use such as bandages and other hemostatic agents (Pusateri et al, 2003). In medical applications, they are clearly understood for the great current interest of chitosan and some of its derivatives. They are investigated for having the capability to interact with lipids (fat) from the digestive system and limit their absorption in the body, approach health benefits (Fukada et al, 1991). Generally, the application potential of chitosan, a deacetylated derivative of chitin, are multifunctional, such as in food and nutrition, biotechnology, material science, drugs and pharmaceuticals, agriculture and environmental protection, the biomedical, food, and chemical industries, and recently in gene therapy too (Prashanth and Tharanathan, 2007; Knorr et al, 1984) (Figure 2). Moreover, products of chitin through enzymatic and acidic hydrolysis processes-derived chitosan and its derivatives such as COS are able to use for potential biological applications because of the properties of the biocompatibility and nontoxic nature of chitosan (Park and Kim 2010). Many reports have showed the hypocholesterolemic and anti-microbial effects and their mechanisms as the research materials advances in other bioactivities from chitosan and COS. In the food industry, edible chitosan possessing more than 83% deacetylation degree and COS have been applied to food science as dietary food additives and functional factors for their effect such as anti-microbial, hypocholesterolemic, and immune regulating activity (Xia et al, 2010). Many commercial functional products are able for use, such as chitosan capsules and COS capsules. It's also reported that edible chitosan biofilm has been used for food storage utilizing its anti-microbial activity (Xia et al, 2010). Plant protection and feed additives have been utilized in agriculture (Kurita et al, 1998). Cosmetics also contribute for one of the most important fields of chitosan (Park and Kim 2010).

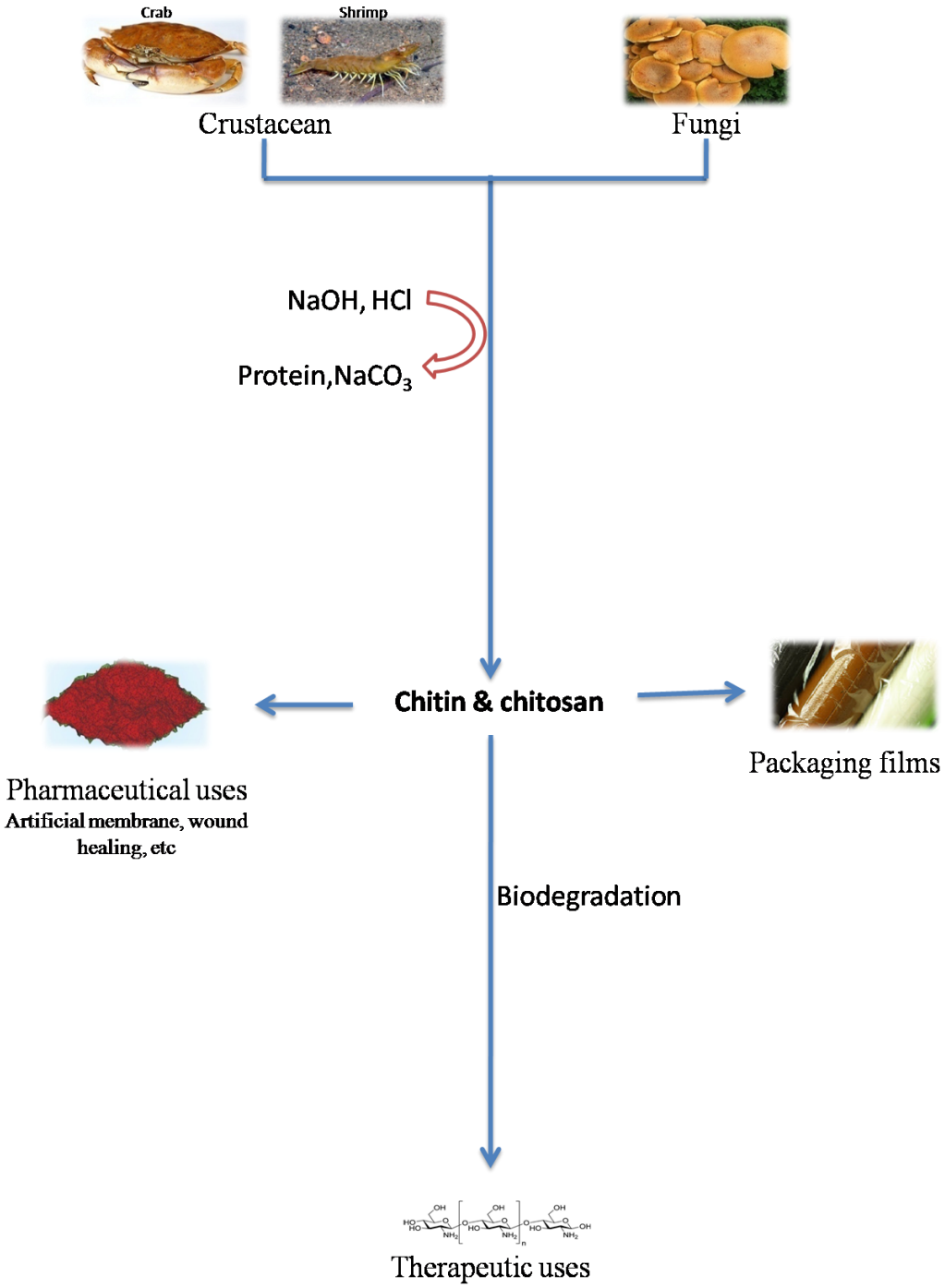


Figure 3. Valuable production of chitin/chitosan

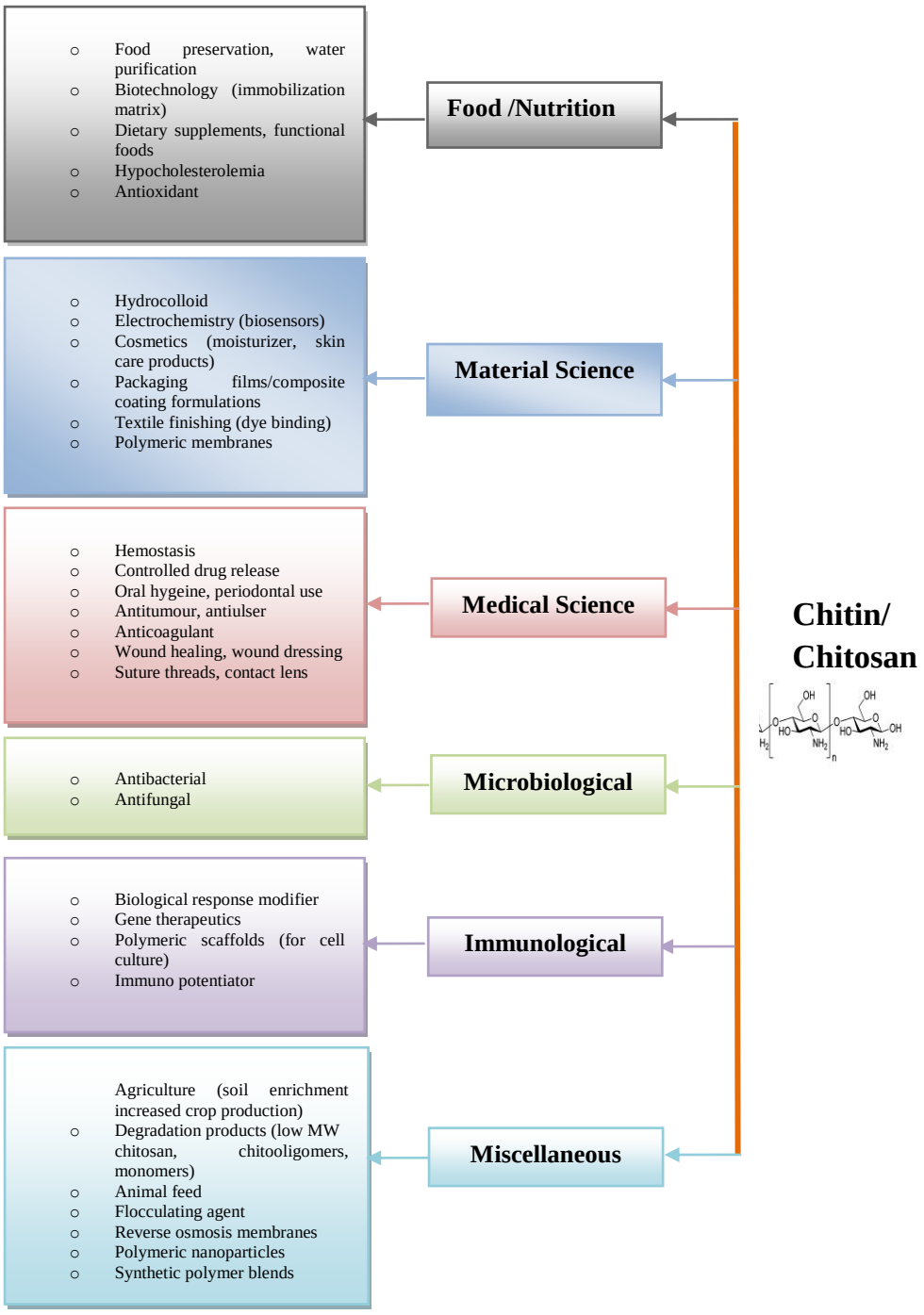


Figure 4. Application potential of chitin/chitosan

There are three types of reactive functional groups in chitosan's polymer, an amino/acetamido group and primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions. The main reason for the differences between their structures and physicochemical properties are the amino contents that are correlated with their chelation, flocculation and biological functions (Xia et al, 2010). Recently, researches on the biological activities of chitosan and its oligomers have been increasing. However, there is no single derivative of chitosan or its oligomers showing expert of all of the above bioactivities. Moreover, different chitosan derivatives and their products have different structures and physicochemical properties. These properties may result in novel bioactivities or novel findings in bioactive compounds (Xia et al, 2010). Many researches have reported the biological activities of chitosan and its derivatives correlated with their structures and physicochemical properties of main polymer and bioactive modified group, including the hypocholesterolemic effects of different chitosan samples on anti-microbial effects, immunity-enhancing, anti-tumor and anti-cancer effects, the acceleration of calcium and iron absorption, and other biological activities such as anti-inflammatory and anti-oxidant activities (Ngo et al, 2011**;** Yasufuku et al. 2010; Sun et al, 2010; Xia et al, 2010; Park and Kim, 2010; Yoon et al, 2009; Anraku et al, 2008; Moon et al, 2007; Rajapakse et al, 2007; Kim et al, 2006; Kim et al, 2004; Lin and Chou, 2004; Vongchan et al, 2002; Jeon et al, 2002; Jia, 2001; Eon et al, 2000; Kondo et al, 2000; Ghaouth et al, 1992) (Table. 1). For instant, one of the crucial activities of chitosan derivatives is anti-oxidant effect. Recently, anti-oxidant activity of chitosan oligosaccharides and its derivatives has attracted much attention due to their nontoxic nature and natural abundance (Sun et al, 2010). Anraku et al. (2008) reported that the scavenging of hydroxyl radicals by LMW chitosan inhibits the peroxidation of human serum albumin (HSA). Yasufuku et al. (2010) also reported the anti-oxidant and free radical-scavenging properties of several LMW chitosan preparations in in vitro studies. He also evaluated the anti-oxidant properties of an extended-release tablet that contained combinations of LMW chitosans. In rats, the administration of chitosan treated with isoniazid or rifampicin was reported to prevent hepatotoxic lipid oxidation (Santhosh et al, 2006). Similarly, it was reported that the injection of chitosan showed the inhibitory effect on glycerol-induced renal oxidative damage in animal rats by Yoon et al. 2008. Sun et al. (2010) reported the anti-oxidant activity of N-acyl chitosan oligosaccharides. He resulted that N-maleoyl chitosan oligosaccharide possess a stronger

electron-withdrawing effect. The anti-oxidant activity of chitosan oligosaccharide and its derivatives were reported that the activity of hydroxyl and amino groups were the important factors as well as involvement of the anti-oxidative effect of chitosan oligosaccharide and its derivatives. The properties of modified groups may result in the anti-oxidant activity of chitosan and its derivatives. The modification can reduce the amount of active amino and hydroxyl groups in the polypolymer chains. They may partly destroy the intermolecular and intramolecular hydrogen bonds. For example, N-maleoyl chitosan oligosaccharide and N-succinyl chitosan oligosaccharide have the same modification degree, and the same content of hydroxyl and amino groups, but their scavenging activity on superoxide radical, hydroxyl radical and reducing power are different. These studies could be demonstrated basing on the activity of hydroxyl and amino groups. The N-maleoyl chitosan oligosaccharide was grafted by -COCH=CH-COO- group and the N-succinyl chitosan oligosaccharide was grafted by -$COCH_2CH_2COO$- group. Both -COCH=CH-COO- group and -$COCH_2CH_2COO$- group are electron-withdrawing groups, the electron-withdrawing effect of -COCH=CH-COO- group and the -$COCH_2CH_2COO$- group can reject the intermolecular and intramolecular hydrogen bonds and support the activity of hydroxyl and amino groups. Furthermore, the electron-withdrawing effect of -COCH=CH-COO- group is stronger than that of -$COCH_2CH_2COO$- group, thus with the same substituting degree, the scavenging effects on superoxide radical, hydroxyl radical and reducing power of N-maleoyl chitosan oligosaccharide were stronger than that of N-succinyl chitosan oligosaccharide. Yasufuku et al. (2010) also reported the anti-oxidant activity of LMW (low molecular weight) chitosan: reduce Cu^{2+}, hydroxyl and superoxide radicals and N-centered radicals. In this year, Ngo et al. (2011) have reported their anti-oxidant activity on decrease of reactive oxygen species induced activation of the nuclear transcription factor NF-κB, free radical-scavenging properties of several LMW chitosan preparations in in vitro studies.

Table 1. Biological activities of chitosan and its derivatives

Derivatives	Activity	Functional properties	Reference
COS	Anti-oxidant activity	Decrease of reactive oxygen species induced activation of the nuclear transcription factor NF-κB, free radical-scavenging properties of several LMW chitosan preparations in in vitro studies	Ngo et al, 2011
(2-pyridyl)-acetyl Chitosan	Anti-oxidant activity	Hydroxyl radicals and superoxide radicals scavenging	Li et al, 2011
Low molecular weight chitosan (38 kDa)	Anti-oxidant activity	The anti-oxidant properties of an extended-release tablet that contained combinations of LMW chitosans: reduce Cu^{2+}, and hydroxyl and superoxide radicals and N-centered radicals	Yasufuku et al, 2010
N-acyl COS	Anti-oxidant activity	Superoxide anion $O_2.^-$, hydroxyl radical $^{\cdot}OH$ and determination of reducing power	Sun et al, 2010
Chitosan and its oligosaccharides	Anti-oxidant activity	Reduction of serum FFA and MDA concentrations and elevate SOD, CAT and GSH-PX activities, the latter being the major anti-oxidant enzymes in the body (in vitro and in vivo).	Xia et al, 2010

Water-soluble chitosan	Anti-microbial effects	Killing of gram-negative bacteria, a cationic chitosan must interact with both bacterial cell envelope membranes. A number of polycationic amines which can interact with the negatively charged residues of carbohydrates, lipids and proteins located on the cell surface of bacteria.	Park and Kim, 2010
COS	Anti-inflammatory activity	The inhibition of TNF-α in the LPS-stimulated inflammation: TNF-α and IL-6 secretion	Yoon et al, 2009
COS	Anti-microbial activity	In vitro and in vivo anti-microbial activity of water-soluble chitosan oligosaccharides against *Vibrio vulnificus*	Lee et al, 2009
Water-soluble Chitosan	Anti-oxidant activity	Inhibition of neutrophil activation and oxidation of serum albumin commonly observed in patients undergoing hemodialysis, resulting in reduction of oxidative stress associated with uremia	Anraku et al, 2008
COS	Anti-bacterial and immunestimulative effect	Against infection by *Staphylococcus aureus* isolated from bovine mastitis	Moon et al, 2007

Carboxylated chitooligosaccharides	Anti-oxidant activity	- Inhibition of lipid and protein oxidation - Inhibition of myeloperoxidase activity in human myeloid cells, suggesting the indirect possibility of inhibiting generation of ROS such as superoxide radicals, H_2O_2 and HOCl. - Reduction of the level of lipid hydroperoxides and carbonyl carbon content in mouse macrophages	Rajapakse et al, 2007
COS	Anti-bacterial and immunestimulative effect	Against infection by *Staphylococcus aureus* isolated from bovine mastitis	Moon et al, 2007
Chitosan sulfates	Anti-oxidant activity	Inhibitory activities of the derivatives toward superoxide radical	Zhong et al, 2007
COS	Anti-inflammatory activity	Inhibition of activation and expression of MMP-2 (matrix metallopeptidase 2) in primary human dermal fibroblasts	Kim et al, 2006
Water-soluble chitosan (20 kDa)	Anti-oxidant activity	The administration of chitosan to rats that had been treated with isoniazid or rifampicin prevented hepatotoxic lipid oxidation	Santhosh et al, 2006

Dimethylaminoethyl-chitosan	Anti-microbial effects	Involve cell lysis: breakdown of the cytoplasmic membrane barrier and the chelation of trace metal cations by the chitosan	Je et al, 2006
Water-soluble chitosan	Anti-inflammatory activity	Inhibition of the secretion of both IL-8 and TNF-α from mast cells, reduced the allergic inflammatory response	Kim et al, 2004
Chitosan and N-alkylated disaccharide	Anti-oxidant activity	Non-toxic offer protection, retarding the progress of numerous chronic diseases, lowered peroxide values and total volatile aldehydes	Lin and Chou, 2004
COS	Anti-tumor activity	Inhibition of the growth of both tumor cells in the mice, against Sarcoma 180 solid (S180) or Uterine cervix carcinoma No. 14 (U14) tumor cell-bearing mice.	Jeon and Kim, 2002
COS	Anti-oxidant activity	The radical scavenging effects on hydroxyl radical, superoxide radical, alkyl radical, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical	Park et al, 2003

COS	Anti-microbial activity	The growth of most bacteria was inhibited. The molecular weight of COS is critical for microorganism inhibition and required higher than 10,000 Da.	Jeon et al, 2002
Sulfated chitosan derivatives	Anti-coagulant activity	Interfering with blood coagulation process. The interaction between the negatively charged sulfated groups and positively charged peptide sequences.	Vongchan et al, 2002
Quaternary N-alkyl chitosa	Anti-microbial activity	A change in cell permeability due to interactions between the positive charges of chitosan and the negative charges on the cell surface. Bind to bacterial genes but also chelate metals such as calcium, magnesium and zinc ions, leading to the inhibition of transcription and translation	Jia, 2001
COS	Acceleration of calcium and iron absorption in vivo	Decreased the excretion of fecal calcium and improved the anti-fracture capacity of rat thighbone	Eon et al, 2000

Low molecular weight of 20 kDa	Anti-diabetes	LMWC of 20 kDa prevents progression of low dose streptozotocin induced slowly progressive diabetes mellitus in mice	Kondo et al, 2000
N-sulfonated and N-sulfobenzoyl chitosan	Anti-microbial effects	The positive charge on the C-2 of the glucosamine monomer at pH values below 6, chitosan is more soluble and has a better anti-microbial activity than chitin dependent on their degree of polymerization (their inhibitory effects increased with increasing DD), molecular weigh (molecular weiSght of COS is critical for microorganism inhibition and required higher than 10,000 Da), pH of the medium, the temperature, the presence of several food components.	Chen et al, 1998
Chitosan	Anti-fungal activity	Induction of morphological and cytological alterations in *Rhizopus stolonifer*	Ghaouth et al, 1992

B. Dendriric cells (DCs)

1. Dendritic cells (DCs) subsets

Dendritic cells (DCs) are antigen-presenting cells forming part of immune cells in the mammalian immune system. Their major is to program antigen material and take it on the surface to other cells of the immune system. In mice, there are two different pathways investigated in mice named myeloid and lymphoid pathway (Banchereau et al, 2000). Lymphoid DCs are different from myeloid in phenotype, localization, and function. However, both subsets show the expression of high levels of CD11c, class II major histocompatibility complex (MHC), and the costimulatory molecules CD86 and CD40. To date, CD8α could use for distinguishing these pathways. CD8α known as a homodimer shows the expression on the lymphoid DCs, but is disappeared from the myeloid (Wu et al, 1998; Pulendran et al, 1997). Lymphoid DCs are in the T cell–rich areas of the periarteriolar lymphatic sheaths (PALS) in the spleen and lymph nodes (Pulendran et al, 1997). In contrast, myeloid DCs are in the marginal zone bridging channels of the spleen. The lymphoid DCs can express greater levels of interleukin IL-12 and are less phagocytic than myeloid DCs (Pulendran et al, 1997). IL-12 is responsible for induce of production of IFN in lymphoid but not in myeloid DCs (Ohteki et al, 1999). In vitro, it was reported that the lymphoid DCs play a function to activate allogeneic CD4 and CD8 T cells less efficiently than the myeloid DCs (Kronin et al, 1996; Suss et al, 1996). In vivo, both lymphoid and myeloid DCs are responsible for priming Ag-specific $CD4^+$ T cells efficiently (Pulendran et al, 1999). In human, DC heterogeneity is manifested at four levels (Banchereau et al, 2000) (Figure 5). Precursor populations are known as the first item. There are two subsets of DC precursors found in the blood: $CD14^+$ $CD11c^+$ monocytes and lineage-negative (LIN^{neg}) CD11c-IL-3Rα$^+$ precursor DCs (Grouard et al, 1997; Olweus et al, 1997). The LIN^{neg} $CD11c^+$ cells are known as the third precursor. The second level is the localization. They participate in a various places including skin epidermal DCs, dermal (interstitial) DCs (intDCs), splenic marginal DCs, T-zone interdigitating cells, germinal-center DCs, thymic DCs, liver DCs, and blood DCs. The third one is function. It is different function between murine and human DCs subsets for regulating B cell proliferation and T cells differentiation towards type 1 or type 2. The final level is determined to the output of immune response. It refers to the induction of immune-tolerance. Totally, there are three subsets of human DCs. Myeloid $CD34^+$ progenitors can differentiate into monocytes ($CD14^+$ $CD11c^+$ DC

precursors) that lead to the immature DCs in response to granulocyte/macrophage colony-stimulating factor positive (GM-CSF), interleukin-4 (IL-4) and also provide macrophages in response to macrophage colony stimulating factor (M-CSF) (the interstitial pathway) (Banchereau et al, 2000). Myeloid progenitors are also responsible for differentiation of $CD11c^+$ $CD14^-$ precursors. This precursors can support Langerhans cells in response to GMCSF and IL-4 and transforming growth factor TGF-β, and macrophages in response to M-CSF. In cultures, the late precursors might differentiate into DCs. The lymphoid $CD34^+$ progenitor is able to generate the $CD14^-$ $CD11c^-$ IL-$3R\alpha^+$ DC precursors. In fetal thymus organ cultures, a blood cell population is responsible for T cells activation. $CD11c^-$IL-$3R\alpha^+$ DC precursors show the function of differentiation into immature DCs in response to IL-3. The immature cells can be differentiated to mature cells in response to cytokines (MCM, monocyte-conditioned medium) or pathogen products (lipopolysaccharide (LPS) or DNA).

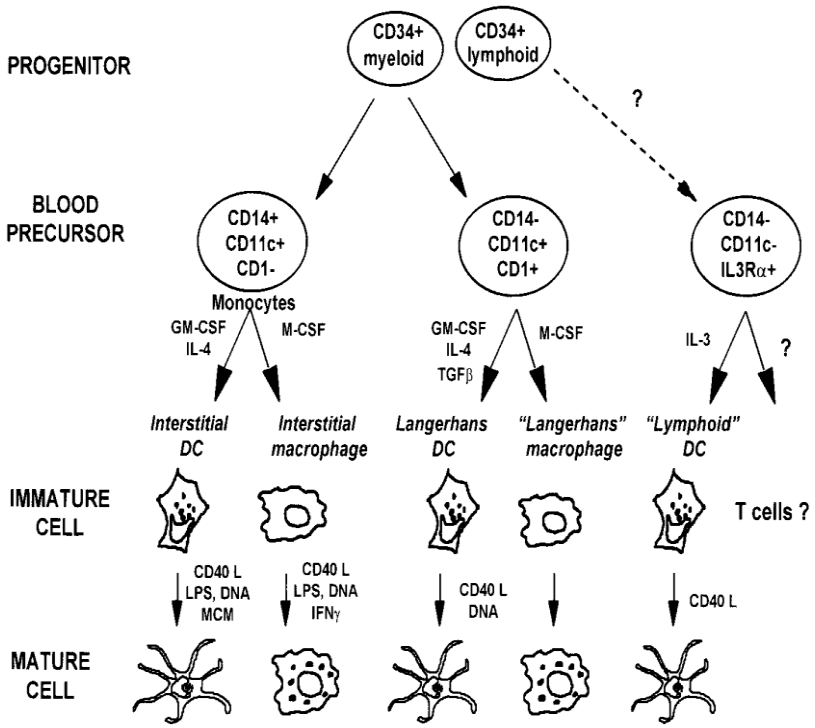


Figure 5. Subsets of human DCs

2. DCs immunology

Host defense immunologically bases on the action of both antigen (Ag)–nonspecific innate immunity and Ag-specific adaptive immunity (Banchereau et al, 2000). The major features of the mammalian innate immune system is requirement of the ability to rapidly detect pathogen or tissue injury and the ability to load the dangerous signal to cells of the adaptive immune system (Banchereau and Steinman, 1998). The innate system contains phagocytic cells, natural killer (NK), cells, complement, and interferons (IFNs) (Bos and Kapsenberg, 1993). It is used for a variety of pattern recognition receptors of cells in the innate system to detect patterns linked between pathogens, for instance bacterial lipopolysaccharide (LPS), carbohydrates, and double-stranded viral RNA (Muzio et al, 1998). Development of adaptive immunity can support by evolutionary pressure. The major features of development of adaptive immunity are the ability to make up genes of the immunoglobulin family, creating a large diversity of Ag-specific clones and immunological memory (Schijns et al, 2000). However, this is too complicated and potent system. They need to be modulated by Ag-presenting cells (APCs). Dendritic cells (DCs) are known as a unique APCs able to induce primary immune responses. They allow development of immunological memory (Steinman, 1991). DC progenitors in the bone marrow can load the signals to induce circulation of precursor. They are in immature status and can show a high phagocytic capacity (Banchereau et al, 2000) (Figure 6). Circulation of precursor DCs can process migration of immature DCs to tissues. Also, they can directly fight pathogens (e.g. viruses) and responsible for secretion of cytokines. These cytokines in turn can activate eosinophils, macrophages (MF), and natural killer (NK) cells. Microbial products can be transmitted through Toll-like receptors (TLRs) and program DCs maturation and then activate T cells. After they capture antigen, DCs can undergo phenotypic and functional changes to differentiate into their mature stage resulting in up-regulation of specific proteins for antigen presentation and T cell activation, including major histocomplex (MHC) and the co-stimulatory molecules CD40, CD80, and CD86, down-regulation of antigen uptake and processing capacity (Yang et al, 2008). The migration of immature DCs is towards to lymphoid organs. After maturation DCs, they can display peptide-major histocompatibility complexes and allow selection of rare circulating antigen-specific lymphocytes. DCs activate T cells to express CD40 ligand. It is very important for DCs-derived cytokines in T cell differentiation into Th_1 or Th_1 phenotype (Aicher et al, 1999).

DCs-secreted IL-12 plays an important role in supporting $CD4^+$T cell differentiation to a Th_1-cytokine pattern, producing IFN-γ, but not Th_2 cytokines such as IL-4 (Aicher et al, 1999). These cytokines activate T cells and help DCs in terminal maturation. They give a permit of lymphocyte expansion and differentiation. Activated T lymphocytes migrate and can reach the injured tissue. Helper T cells secrete cytokines and allow activation of macrophages, NK cells, and eosinophils. Cytotoxic T cells eventually digest the infected cells. B cells go to activation after interact with T cells and DCs. Then they migrate into various areas. They mature into plasma cells and produce antibodies that neutralize the pathogen. After interaction with lymphocytes, DCs die by apoptosis.

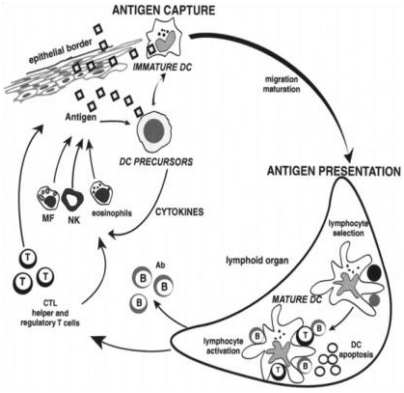


Figure 6. Circulation and presentation of DCs

Perceiving of pathogens is an important step in launching an adequate immune response. The immune system has associated with a complex system of pathogen in which pathogen recognition receptors-called specialized molecules and dendritic cells-like specialized cell types play important majors (Berthier et al, 2000). Dendritic cells (DCs) are very important antigen-presenting cells. Upon stimulation by danger signals of external pathogens and abnormal cells, DCs enroll into activation/maturation to an active antigen-presenting phenotype (Gallucci et al, 2007). The molecules on the cell surface of antigen-laden signal 1-called MHC class I/II molecules, up-regulation of costimulatory membrane proteins, such as signal 2- called CD80 and CD86, and signal 3-called secretion of proinflammatory cytokines (TNF-α, IL-6, or IL-12) are responsible for this phenotype (Behrens et al, 2007). Recent researches have reported that all these signals are responsible for T lymphocyte activation. In enxogenous

pathway, DCs can transfer MHC class I–loaded antigens through this pathway with peptides originating from phagocytosed particulate antigens or immune complexes (Figure 7). Peptides are produced in the proteasome, transferred into the endoplasmic reticulum (ER), and loaded onto the nascent MHC class I molecules.

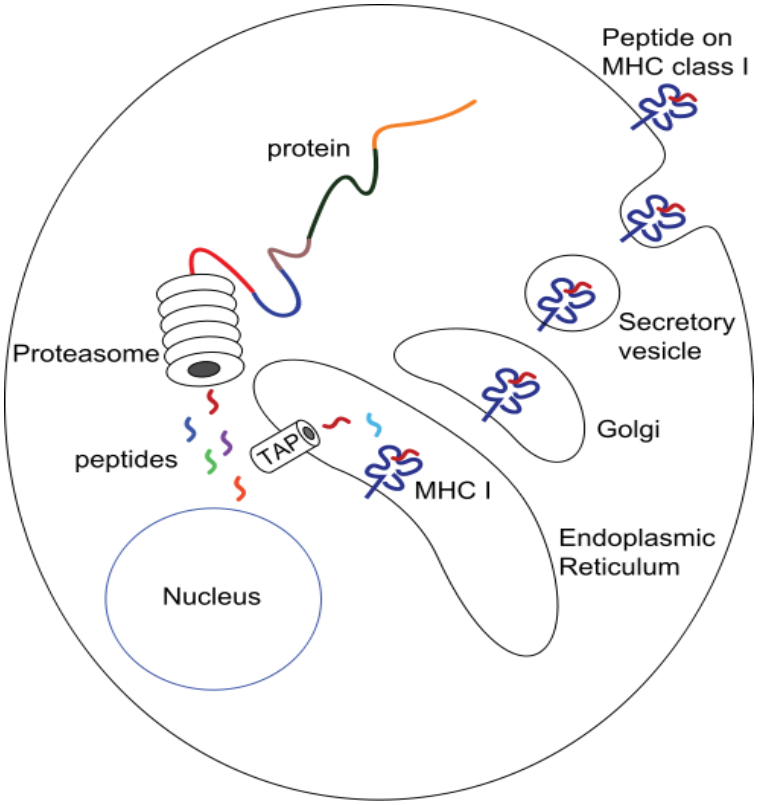


Figure 7. MHC class I pathway

On the other hand, MHC class II, DCs can uptake soluble antigen (Ag) either through macropinocytosis or receptor-mediated endocytosis (Figure 8). They can also uptake particles through phagocytosis. The antigens go to degradation in endosomes. They are produced polypeptides and then transported into the MHC class II–rich compartments for their loading onto the nascent MHC class II molecules. The peptides are transferred onto empty MHC class II molecules recycled from the cell surface.

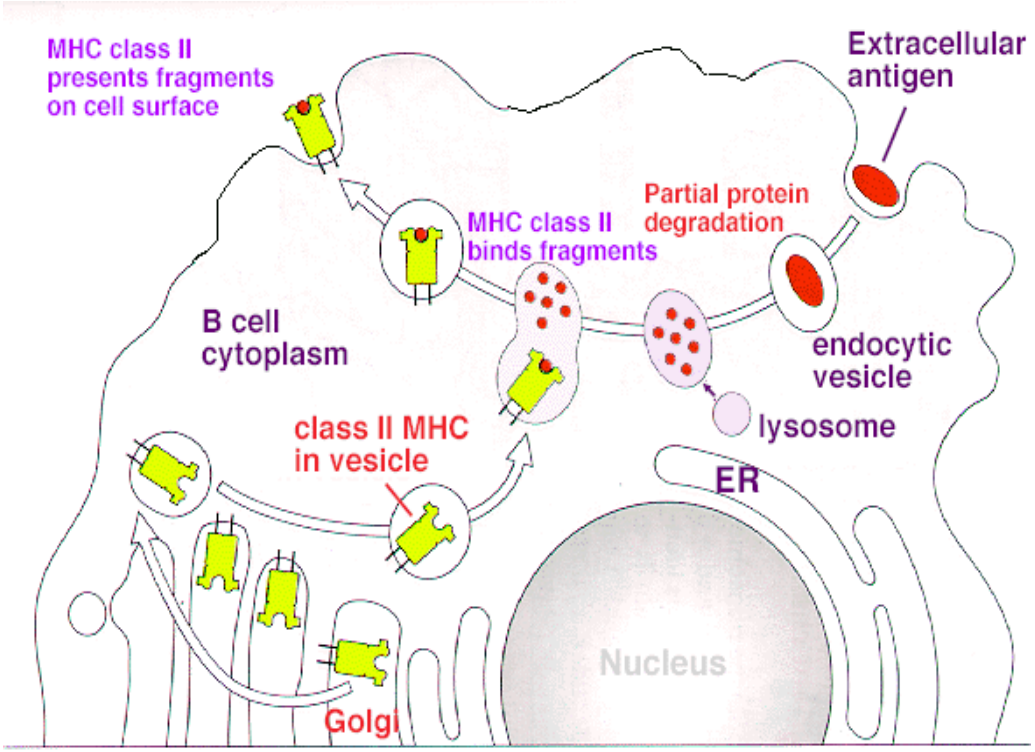


Figure 8. MHC class II pathway

Most danger signals are perceived through receptors belonging to the Toll-like receptor (TLR) family (Behrens et al, 2007; Murray et al 2007; Kennedy et al, 2004). They can interact with a wide range of ligands from bacterial products (e.g. glycolipids andlipopolysaccharides) or viral products (e.g. double-stranded RNA) (Goetz et al, 2000) (Figure 9). However, a few endogenous TLR ligands are also known and may expose part of the behavior of DCs towards endogenous activation signals (Gallucci et al, 1999). Additionally, danger signals coming from eukaryotic pathogens is unclearly understood (e.g. fungi, protozoa, etc.). They are perceived by DCs. TLRs is referred in the perceiving of fungal polysaccharides by monocytes and DCs (Behrens et al, 2007). There are very few reports focusing on the involvement of TLRs in fungal polysaccharide recognition. Moreover there are several pathways for polysaccharide sensing, approaching to different DC phenotypes (Gallucci et al, 1999). Furthermore, there is a close relationship between sensing of danger signals sometimes and the molecules other than the ligands and TLRs (Verhasselt et al, 1997).

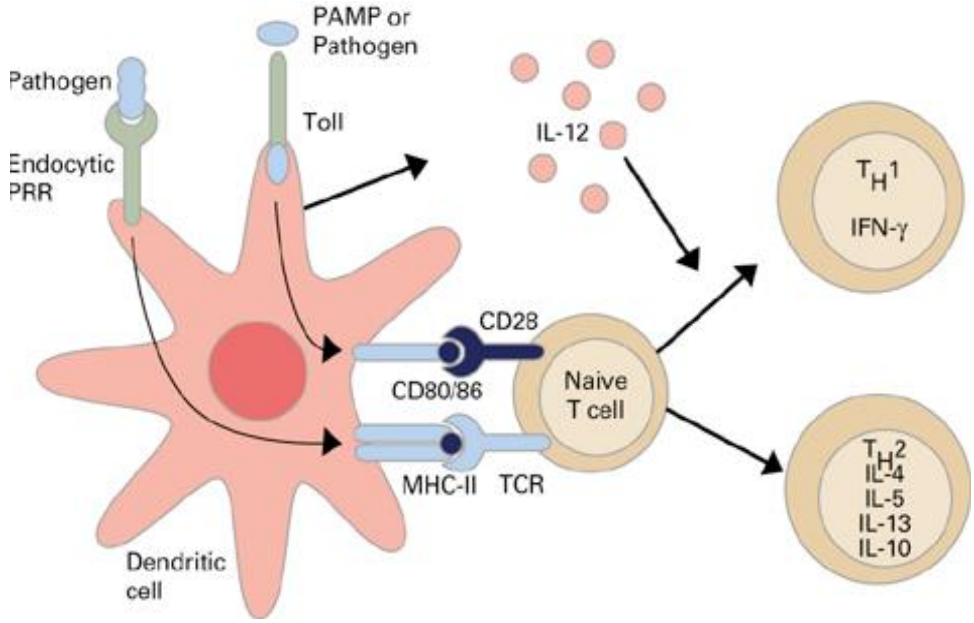


Figure 9. Angiten presenting function of DCs-linked TLR

There are several molecules such as CD40, TNF-R, and IL-1R responsible for activation of DCs and their transition from the immature cells to mature DCs. In the periphery, DCs still process their maturation during the DC–T cell interaction. The numerous factors such as pathogen-related molecules of LPS, bacterial DNA, and double-stranded RNA can induce and regulate DCs maturation (Figure 4). The balance between pro-inflammatory and anti-inflammatory signals in the local microenvironment, including TNF, IL-1, IL-6, IL-10, TGF-β, and prostaglandins; and T cell–derived signals are also able to regulate DCs maturation **(**Rescigno et al, 1998; De Saint-Vis et al, 1998; Foey et al, 1998**)**. It is associated with several coordinated events for the maturation DCs such as loss of endocytic/phagocytic receptors; up-regulation of costimulatory molecules CD40, CD58, CD80, and CD86 (Murray et al, 2007); change in morphology, movement in lysosomal compartments with down-regulation of CD68 and upregulation of membrane protein (DC-LAMP), and change in class-II MHC compartments (Saint-Vis et al, 1998; Rescigno et al, 1998). The antigen/pathogen is responsible for induces of the immature DC to undergo phenotypic and functional changes. DC maturation is intimately connected to their migration from the peripheral tissue to the draining lymphoid organs.

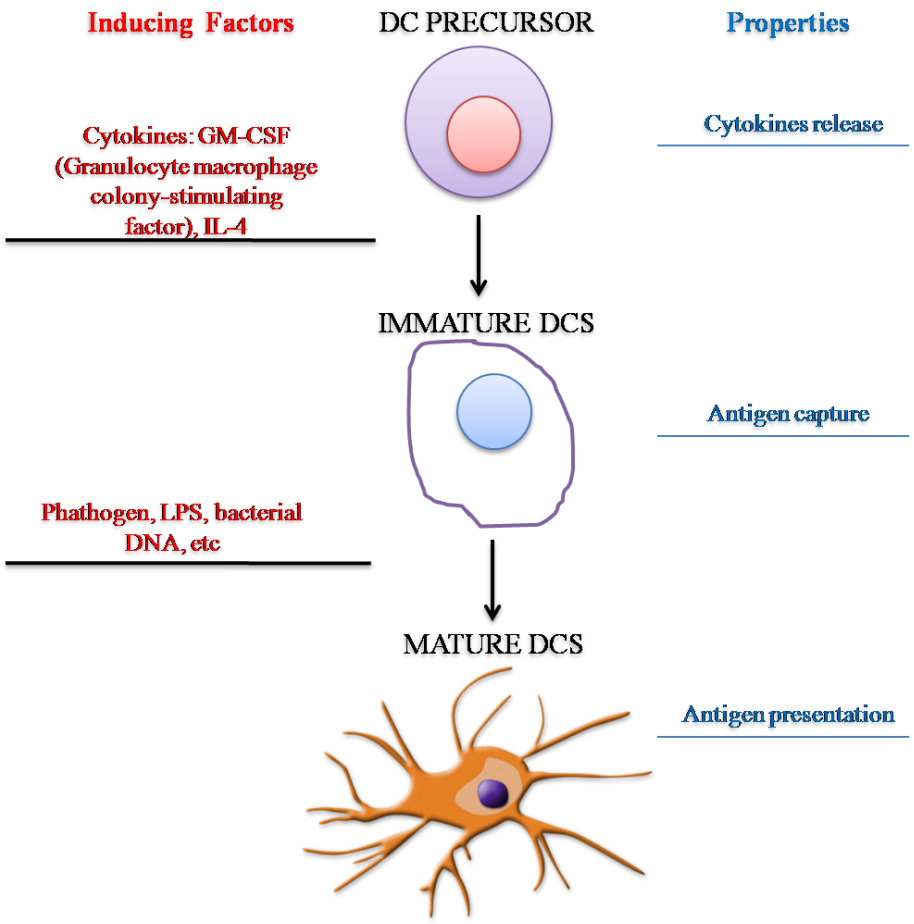


Figure 10. Maturation of DCs

MAPK family members are very important for the signal transduction of a variety of cellular functions (Craxton et al, 1998). P38 MAPK pathway is needed for CD40-induced proliferation in human tonsillar B cells (Alexandra et al, 1999). Bacterial constituents such as LPS, proinflammatory cytokines, and ligation through TNF receptor (TNFR) family members, including CD40, can activate p38 MAPK (Foey et al, 1998). Immature monocyte-derived DCs undergo differentiation into mature DCs upon stimulation with LPS (Nakahara et al, 2004). Cell proliferation and survival is involved the ERK pathway (Rescigno et al, 1998). In a murine DC cell line, ERK is known as a survival factor (Rescigno et al, 1998). Several transcription factors, such as ATF-1, ATF-2, and cAMP response element binding protein, are also regulated

via a p38 MAPK-dependent pathway in response to certain stimuli (Raingeaud et al, 1996; Tan et al, 1996).

C. Immunotherapeutic chitosan

In animals, the non-specific immune system is responsible for initiating and general responses against invasion of microorganisms. Immunostimulants are generally identified as compounds. They can modulate the non-specific immune system by improving the capability of defending activity of phagocytes. Almost immunostimulants can specifically bind with the cell surface receptor proteins of phagocytes or lymphocytes. They stimulate the production of immune response compounds such as interferon, interleukin and complement proteins that activate the immune system. In addition, some immunostimulants are able to compete with specific receptor molecules in the target cells of infectious organisms. It's also reported the effect of carbohydrate derivatives including mannan oligosaccharides, peptidoglycan and COS on immunoregulation (Matsuo and Miyazono, 1993). Recently, chitosan and their formulations have been reported to have immune stimulating activity such as increasing accumulation and activation of augmenting antibody responses and delaying type hypersensitivity (DTH) and cytotoxic T lymphocyte (CTL) responses (Seferian and Martinez, 2002, Wen et al, 2011). Oligomers of chitin and chitosan had reported their effective major in enhancing immune system (Okamoto et al, 2003). Further, water-soluble chitosan can activate the production of cytokines such as interleukin-1 (IL-1β), tumor necrosis factor-alpha (TNF-α), and reactive oxygen intermediates to promote the defense system against microbial infections (Feng et al, 2004). Additionally, chitosan has been reported their effect on immune system involved to the formulations for utilizing in transmucosal delivery of drugs and peptides. Chitosan and their formulations can be taken up by phagocytotic cells inducing strong systemic and mucosal immune responses against antigens (Lubben et al, 2001). Besides modulating the immune response by stimulating the capture by phagocytotic cells, they may also stimulate the immune system (Illum et al, 2001) (Table. 2). Recently, chitosan and its derivatives-based nanoparticle vaccines have been investigated as adjuvants to antigen-presenting cells (APCs) (Borges et al, 2008). Adjuvants can consider as substances or devices. They can enhance the delivery or immunogenicity of an antigen. The improvement of delivery can effect on the localization

time of the antigen at the site of action and increase immunogenicity and activation of antigen presenting cells (APCs), especially DCs (Gallucci et al, 1999) .

In recent years, chitosan was revealed that the tumor inhibitory effect of its derivatives is probably related to their induction of lymphocyte cytokines through increasing T-cell proliferation. Basically, the acquired immunity via accelerating T-cell differentiation to increase cytotoxicity and maintain T-cell activity can be enhanced by investigating the anti-tumor mechanism of COS. The results display that the low-molecular-weight water-soluble chitosans and oligochitosans may possess the useful characters in preventing tumor growth. Their function demonstrated on enhancing cytotoxic activity against tumors as an immunomodulator (Park and Kim, 2010) (Figure 10). In addition, they activate macrophages via the production of cytokines such as interferon (IFN)-γ, interleukin (IL)-12 or -18 from the intraepithelial lymphocytes (Park and Kim, 2010). Chitosan derivatives was examined for the anti-tumor effects of various low-molecular weight chitosans, such as water-soluble 21- or 46-kDa molecules with low viscosity which displayed the decrease of tumor growth and final tumor weight in sarcoma 180-bearing mice due to increase of natural killer cell activity (Maeda and Kimura, 2004). One research group observed that cancer-cell viability was significantly reduced due to the positive or negative charge of different COS derivatives. Moreover, COS was reported inhibition of human hepatocellular (HepG2) carcinoma cell proliferation and down-regulation of cell cycle-related gene expressions with decreased DNA content and up-regulation of p21 in vitro (Huang et al, 2006). In in vivo observations, COS inhibited tumor growth (Shen et al, 2009). Other chemically modified structures, aminoderivatized COSs, such as aminoethyl-, dimethyl aminoethyl- and diethyl aminoethyl-COS, showed the induction of cell death and the inhibition of proliferation of human gastric adenocarcinoma cells (Karagozlu et al, 2010). This report showed that water-soluble aminoderivatized COS might concern as the valuable cancer chemopreventive agent (Wu et al, 2010). COS also suppressed tumor angiogenesis in vivo and in vitro through blocking migration of endothelial cells induced by nitric oxide (Prashanth et al, 2005; Lee et al, 2005; Cooke et al, 2003) (Table. 3). That could conclude that structures and physicochemical properties of main polymer and bioactive modified group significantly possess different effects in immune system.

Table 2. Stimulation of chitosan and their derivatives in DCs

Derivatives	Cytokines	Surface Markers	T Cell Stimulation	Author
Chitosan and its nanoparticles	Induce IL-12, IL-10	Uninvestigated	Th_1 (IL-2 and IFN-γ) ; Th_2 (IL-10) Cytokines; IL-2, IFN-γ and IL-10 (Splenocyte in vivo)	Wen et al, 2011
N-trimethyl Chitosan	Induce IL-6 and IL-12	MH Class II, CD83, CD86	IL-4 Than IFN-γ toward Th_2	Suzanne et al, 2010
Chitosan	IL-12, IL-10, IL-1, and TNF, and IL-6 Are Not Induced	Induce Class II, CD80 And CD86	Chitosan + LPS Induced: - T-Cell Proliferation & $CD69^+$ - Cytokines During T Cell Activation (IL-2, IL-6, IL-2, IFN-ɣ, IL-10) - IL-10 & IL-12 in Dose Dependent Change.	Villiers et al, 2009
Chitosan particles	Uninvestigated	Uninvestigated	Increase $CD4^+$ T-Cell Proliferation & IFN-γ/IL-10	Saint-Lu et al, 2009
Polyethylenimine and galactosylated chitosan	Induce IL-12/p70 and IFN-γ	Uninvestigated	Uninvestigated	Kim et al, 2007

Table.3. Immuno-stimulating and anti-tumor effects

Derivative	Biochemical significance	Author
LMW	Low-molecular weight chitosans, such as water-soluble 21- or 46-kDa molecules with low viscosity, produced by enzymatic hydrolysis of over 650-kDa chitosan, which displayed decreased tumor growth and final tumor weight. Activated macrophages via the production of cytokines such as interferon (IFN)-γ, interleukin (IL)-12 or -18 from the intraepithelial lymphocytes	Maeda and Kimura, 2004
COS	Inhibition of human hepatocellular (HepG2) carcinoma cell proliferation and down-regulated cell cycle-related gene expressions with decreased DNA content and up-regulation of p21 *in vitro*	Huang et al, 2006
COS	Inhibition of lung tumor and lung metastasis	Shen et al, 2009
Aminoethyl-, dimethyl aminoethyl- and diethyl aminoethyl-COS	Inhibition of the proliferation of human gastric adenocarcinoma cells	Karagozlu et al, 2010
COS	Blocking migration of endothelial cells induced by nitric oxide	Wu et al, 2010
LMW and COS	Inhibition of angiogenesis and inducing apoptosis as a function of DNA fragmentation.	Prashanth et al, 2005
Chitosan nanoparticles	Effects on the proliferation of human gastric carcinoma: necrotic cell morphology and DNA fragmentation, Changes of mitochondrial membrane potential	Qi et al, 2005

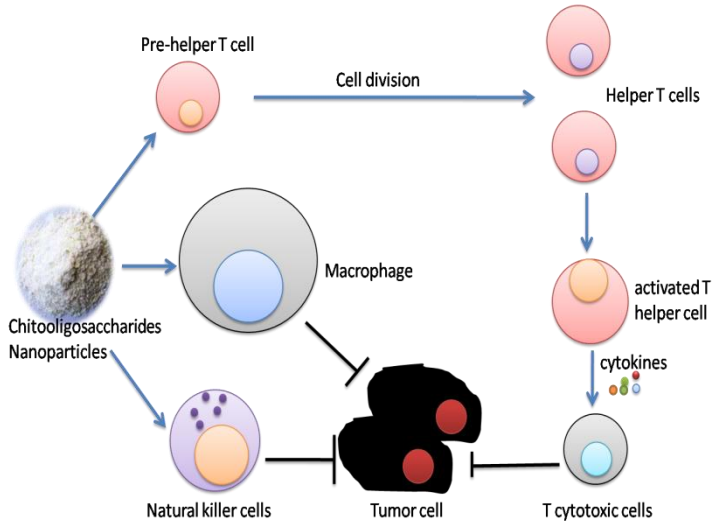


Fig 11. Immunomodulation activity of COS and nano-particles

Chitosan and its products (microparticles, nanoparticles, and its mixture with ovalbumin) have been reported to have immune-stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cells. They can regulate DCs maturation and development through the modulation of the surface markers such as class II, CD80 and CD86 and chitosan-stimulated DCs-released cytokines such as IL-12, TNF-α, and IFN-γ. Therefore, the use of chitosan and its derivatives used as immunological adjuvants to regulate both humoral and cell-mediated immunity is promising. Furthermore, different chitosan derivatives have showed different activities as well as their effects in immune system. In this study, we could investigate the regulation of chitosan (CS) and its derivative named chitosan-catechin conjugate (CSC) on murine monocyte-derived DCs maturation.

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II. MATERIALS AND METHODS

A. Preparation of animals and cell line

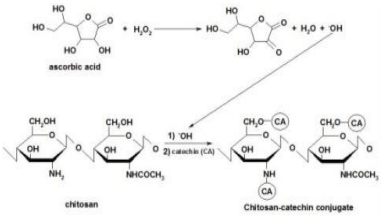
Male 6-8 week-old C57BL/6 (H-2Kb and I-Ab) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). The mice were housed in a pathogen-free environment within our animal facility for at least 1 week before use and were used in accordance with the institutional guidelines for animal care. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1,000 U/ml Penicillin, 50μg/ml streptomycin, and 10% fetal bovine serum (FBS). Cells were maintained at 37^{o}C in 5% CO_2.

B. Reagents and antibodies

Recombinant mouse (rm) GM-CSF and rmIL-4 were purchased from R&D Systems. Dextran- FITC (molecular mass 40,000), and LPS (from Escherichia coli 055:B5) were obtained from Sigma-Aldrich. An endotoxin filter (END-X) and endotoxin removal resin (END-X B15) were acquired from Associates of Cape Cod. Cytokine ELISA kits were purchased from BD PharMingen. FITC- or PE-conjugated monoclonal antibodies (mAbs) used for flow cytometry were purchased from BD PharMingen. To detect protein levels by western blotting, anti-phospho-ERK, anti-ERK, anti-phospho-p38, and anti-p38 were purchased from Cell Signaling. Anti-p65 Ab was from Abcam. 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma.

C. Preparation of chitosan-catechin conjugate (CSC)

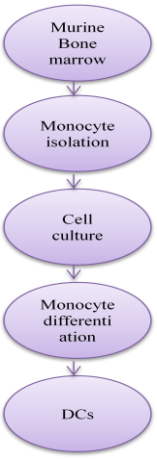
The chitosan (CS) was kindly donated by Kitto Life Co. (Seoul, Korea). The average molecular weight and degree of deacetylation were 310 kDa and 90%, respectively. Briefly, 0.25 g of chitosan was dissolved in 25 mL of 2% acetic acid. Then, 0.5 mL of 1.0 M H_2O_2 containing 0.054 g of ascorbic acid was added. After 30 min, catechin was added and incubated at room temperature for 24 h. Next, the mixture was dialyzed against water for another 48 h. The molar ratio of chitosan units and catechin was 1: 0.1 for CSC. ^{1}H NMR spectrometry and DSC analysis were carried out for identification of CSC.



Scheme 2. Synthesis pathway of chitosan-catechin conjugate

D. Generation and culture of DCs

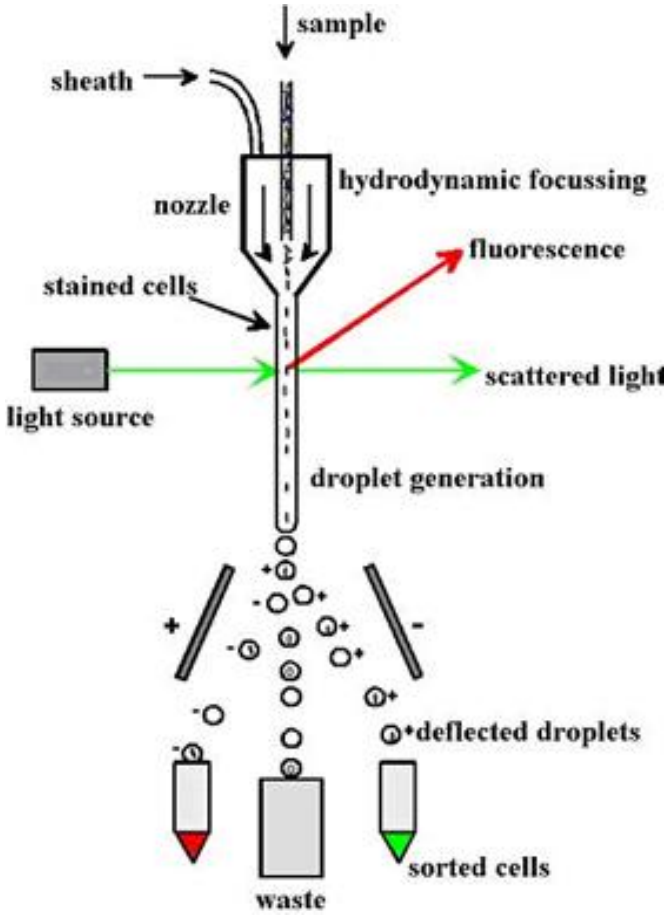
DCs were generated from murine whole bone marrow (BM) cells. Briefly, BM was flushed from the tibiae and femurs of C57BL/6 and BALB/c mice and depleted red cells with ammo-nium chloride. The cells were plated in 6-well culture plates (10^6 cells/ml, 2 ml/well) and cultured at 37°C in 5% CO_2 and OptiMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-gluta-mine, 100 U/ml penicillin, 100μg/ml streptomycin, 5×10^{-5} M β-mercaptoethanol, 10 mM HEPES (pH 7.4), 20 ng/ml rmGM-CSF, and rmIL-4. On day 2 of culture, floating cells were gently removed and fresh medium was added. On day 6, non-adherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation, or in some experiments. On day 6, 80% or more of the non-adherent cells expressed CD11c.



Scheme 3. Basic steps for DCs isolation

E. Flow cytometry

On day 6, BM-DCs were harvested, washed with PBS, and resuspended in fluorescence activated cell sorter (FACS) washing buffer (2% FBS and 0.1% sodium azide in PBS). The cells were blocked with 10% (v/v) normal goat serum for 15 min at 4°C, and stained with phycoerythrin (PE)-conjugated anti- H-2Kb [major histocompatibility complex (MHC) class I], anti-I-Ab (MHC class II), anti-CD40, anti-TLR4, anti-CD80, and anti-CD86 with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (PharMingen, San Diego, CA) for 30 min at 4°C. The stained cells were analyzed using a FACSCalibur flow cy-tometer (Becton Dickinson, San Jose, CA).



Scheme 4. Flow cytometry principle

F. The affect of CSC on cell viability

The concentration of cells were 5×10^4 cells/well of 96 well culture plates for MTT assay MTT assay was performed as described the previous reference (Kim et al, 2008).

G. Determination of cytokines expression by real-time PCR

Real-time PCR was performed using a BioRad MiniOpticon System (BioRad Laboratories, Ltd) with SYBR green. The method follows procedure by Lee, et al. 2011. Reactions were performed in a total volume of 20μl-including 10μl 2x SYBR Green PCR Master Mix (Applied Biosystems), 1μl of each primer at 10μM concentration and 1μl of the reverse-transcribed cDNA template. The cycling protocols was as follows: denaturation (95℃ for 10 min), amplification repeated 40 times (95℃ for 30 s, 52℃ for 30 s, 72℃ for 30 s, and acquisition temperature for 15 s).

III. RESULTS AND DISCUSSION

A. Determination of catechin content in CSC

The method based on the procedure above. The contents of catechin in the conjugate were calculated following the catechin standart curve. Contents of catechin is 23.53 ± 2.38 mg/g chitosan-catechin conjugate (Figure 12).

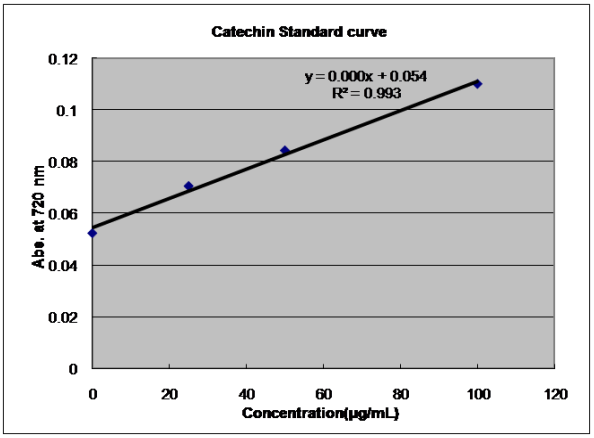


Figure 12. Catechin standard curve

B. Differential scanning calorimetry (DSC)

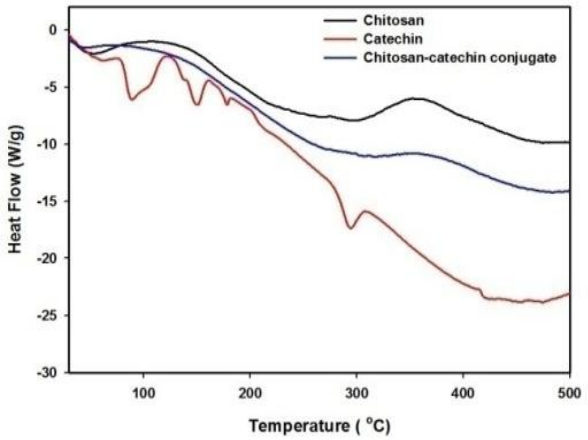


Figure 13. Differential scanning calorimetry (DSC)

DSC is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. DSC is used widely for examining polymers to check their composition. The black, blue, and red line subsequently indicates to chitosan, chitosan-catechin conjugate, and catechin. The value of heat flow showed that chitosan-cetachin is among chitosan and catechin (Figure 13).

C. Cell viability

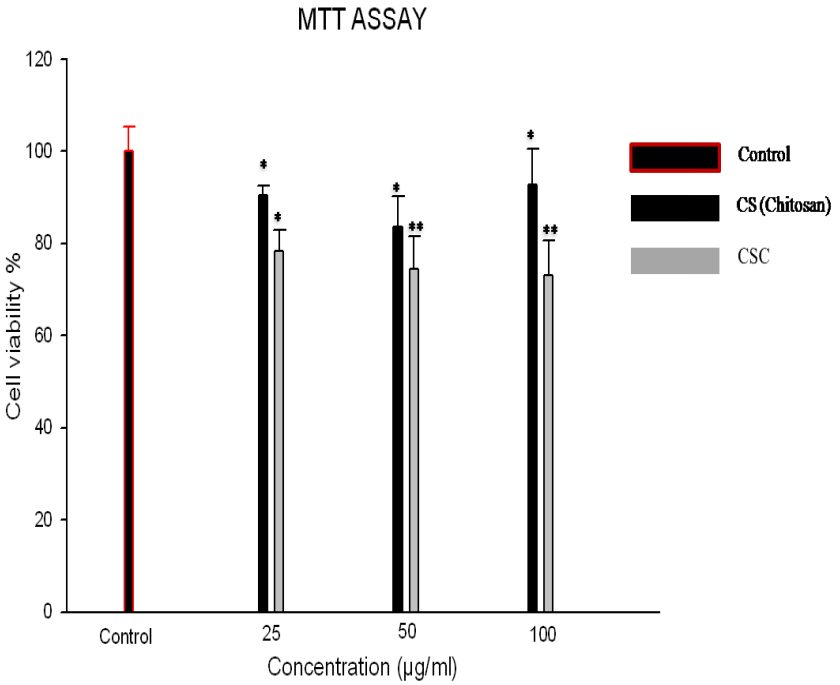


Figure 14. MTT assay. The procedure followed the method above. ** Indicate different significance at $p < 0.05$ compared with control; * Indicate no different

MTT assay were used to determine if CS (chitosan) and CSC (chitosan-catechin) treatment affect the viability of DCs. MTT assay showed that the viability of CS-treated DCs did not affect in cell viability at all the concentrations. MTT assay also showed that the viability of CSC-treated DCs did not affect in cell viability at the concentration less than 50 µg/mL (Figure 14)

D. Effect of CSC on maturation of bone marrow-derived DCs

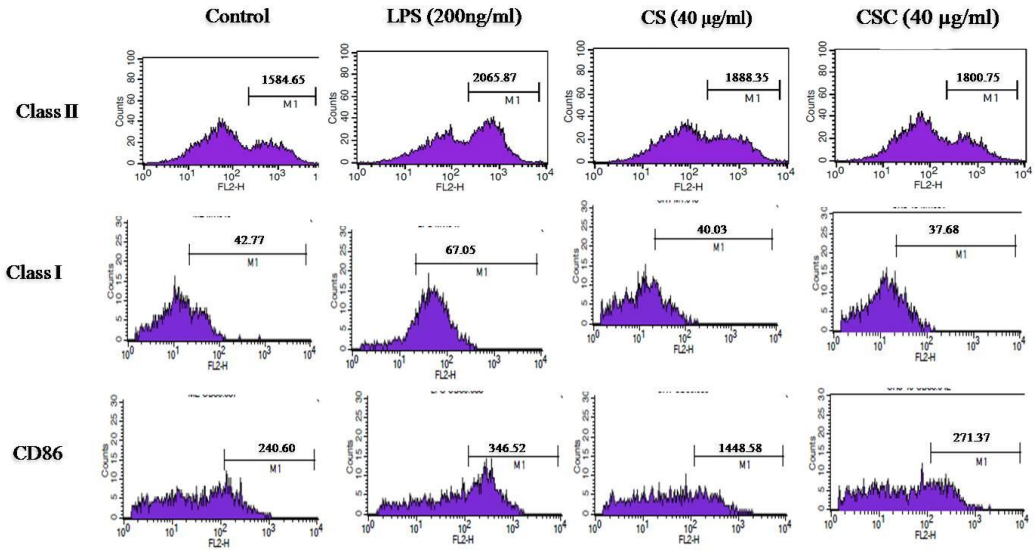


Figure 15. Effect of CSC on maturation of bone marrow-derived DCs

Firstly, we investigated the effects of CS and CSC on the maturation of DCs. Bone marrow-derived DCs were cultured in medium supplemented with granulocyte macrophage colony-stimulating factor (20ng/ml) for six days. Different concentrations of the samples were added on day 6 and lipopolysaccharide (LPS, 200ng/ml)) was used as a positive control. We investigated the effects of a range of the samples concentrations on DC maturation. The DC populations were subsequently analyzed by flow cytometry for expression of cell surface molecules such as CD80 and CD86, as well as MHC class I and II (Figure 15). Expression of these molecules on DCs increased in response to CS treatment and was similar to the response to LPS treatment. On the other hand, untreated DCs retained an immature phenotype (Figure 14). Surprisingly, CSC treatment showed the depression of CD 86 and class I, and less high expression of Class II in comparison with control. SCS may concern towards the negative impact on DCs maturation or the less functional effect on DCs maturation.

E. Determination of cytokines expression by real-time PCR

DCs maturation is familiar with the production of cytokines. In this experiment, we could investigate the expression of cytokines in DCs. Interestingly, cytokines expression (IL-1,

IL-6, and IL-12b(p40) was depressed by stimuli with CSC of 40 μg/ml in comparison with medium (Figure 16). This result looks relevant with the result of examination of expression of CD 86 and class I. It may suggest that SCS treatment could regulate the negative impact on immature DCs.

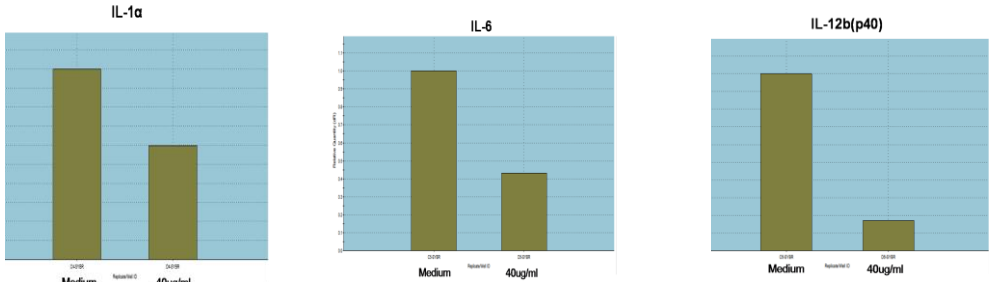


Figure 16. Effects of CSC on cytokines expression

IV. CONCLUSION

In our study, we sought to investigate the modulation of CS and CSC on DC maturation. The DCs maturation by CS or CSC treatment might be an important study to investigate their further effect as an effective adjuvant for DC-based tumor therapy and to evaluate other mechanism involved. In primary investigation, we demonstrated the regulation of CS and CSC in immature DCs via the expression of co-stimulatory molecules production and cytokines expression. According the result, SC stimuli was able to enhance expression of the surface molecules responsible for DCs maturation. Oppositely, SCS treatment showed the negative impact on DCs maturation and activation. It may conclude that the effects of CS and CSC are different on DCs activation. SCS depressed the pro-inflammatory cytokines in immature DCs in comparison with control. In the conclusion, we suggest that CSC could regulate the immature DCs towards the negative modulation.

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