Anti-inflammatory response of activated macrophages using lipid based formulation and its synergy with antioxidant toward the healing of inflammatory diseases

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ANTI-IFLAMMATORY RESONSE OF ACTIVATED MACROPHAGES USING LIPID BASED FORMULATION AND ITS SYNERGY WITH ANTIOXIDANT TOWARD THE HEALING OF INFLAMMATORY DISEASES

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By

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九州大学

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GRADUATE SCHOOL OF SYSTEMS LIFE SCIENCES

KYUSHU UNIVERSITY

Fukuoka, Japan

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ABSTRACT

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Controlling inflammatory response is crucial to avoid the chronic inflammation which leads to severe diseases including neuroinflammation and allergic syndromes such as atopic dermatitis or ulcerative colitis. Macrophage plays a major role for controlling the inflammatory response in human immune system. Conversion of macrophage phenotype from pro-inflammatory to anti-inflammatory is important for the fundamental healing of such inflammatory diseases. The goal of this study was to design nanoparticles loaded simultaneously with both antioxidant and anti-inflammatory molecules targeting the phenotype of macrophages. Thus, we used a combination of α-tocopherol and phophatidylserine containing nanoparticles for resolving the inflammatory response of macrophages.

In chapter 2, I introduced α-tocopherol (T) and phosphatidylserine (PS) containing liposomes (PST-liposomes) to inhibit inflammatory response of microglia, a residential

macrophage of brain. PS is known to have anti-inflammatory effects on microglia, while α-tocopherol is an antioxidant, known to neutralize ROS. We found that both PS-containing liposomes (PS-liposomes) and PST-liposomes, as compared with phosphatidylcholine containing liposomes, significantly increased viability of hypoxia-treated microglia derived cell line. The PST-liposomes functioned better than the PS-liposomes and I attribute this superior effect to a synergy between PS and α-tocopherol. This synergic action of PST-liposomes was illustrated in their ability, when incubated with microglia, to reduce NO and pro-inflammatory cytokine (TNF-α) production and increase anti-inflammatory cytokine (TGF-β1) production. The improved viability of hypoxia-treated microglia when treated with PST-liposomes involved anti-inflammatory effects, including ROS neutralization, as well as induction of a microglial phenotypic change. This results suggest that PST-liposomes will represent a potential therapeutic approach for reducing ischemic injury in brain.

In chapter 3, a phosphatidylserine (PS)-coated microparticle was prepared instead of liposome and tried to apply to AD mouse model for achieving the modulation of the macrophage phenotype to an anti-inflammatory state. Here I prepared poly (D, L-lactate) microparticle coated with PS on the outside shell. I confirmed the cellular uptake of the PS-coated microparticle, which leads to the significant down regulation of the inflammatory cytokine production. In the mouse model of AD, the PS-coated microparticle showed significant reduction in development of AD symptoms comparing with the mice treated with the PC-coated microparticle with subcutaneous injection.

In chapter 4, preparation of potential anti-oxidative and inflammatory property-bearing biodegradable polymer-lipid hybrid nanoparticle (PLNP) containing both α-tocopherol and phosphatidylserine was reported. Then their consequent up-regulation of anti-oxidative and anti-inflammatory response was investigated in activated mouse peritoneal

macrophages.These two components showed a clear synergistic anti-inflammatory and anti-oxidative effects compared with those having only α-tocopherol or PS. Compared with PC-, PCT- or PS-PLNP, the PST-PLNP significantly increased the viability of macrophages. Our data also demonstrated that PCT-, PS- and PST-PLNP significantly suppress the production of NO and inflammatory cytokine, with highest effect showed by PST-PLNP when those cells are stimulated. The PST-PLNP performed better than the PCT- and PS-PLNP and I attribute this highest effect resulted from a synergy between α-tocopherol and PS. The PST-PLNP also showed their anti-inflammatory effect by the production of anti-inflammatory cytokine TGF-β1 in activated macrophages. The potential anti-inflammatory and anti-oxidative effects of the PST-PLNP indicates the phenotypic modulation of the macrophages. Therefore, this α-tocopherol and phosphatidylserine containing polymer-lipid hybrid nanoparticle could be a potential drug carrier and effective approach for healing the chronic inflammatory diseases such as ulcerative colitis.

In chapter 5, the contribution of this research was summarized.

CONTENTS

CHAPTER 1

General introduction

The cell is the basic structural, functional, and biological unit of all known living organisms which performs several functions throughout the life. One of the important functions of cell is to make host defense system of the body against the foreign particles. The cells responsible for the host defense are phagocytes, dendritic cells, lymphocytes and various other leukocytes. Macrophage is one of the specific phagocytes that plays an essentially critical role in non-specific defense as well as specific defense mechanisms by accompanying with antibody or by recruiting other immune cells of the human body. Beyond increasing inflammation and stimulating the immune system, macrophages also play an important anti-inflammatory role and can decrease immune reactions through the release of cytokines [1]. Thus, the control of macrophage phenotype is a key factor for combating the inflammatory diseases.

In this thesis we focus on the functional modulation of macrophage phenotype as from pro-inflammatory to anti-inflammatory response of the cell by the secretion of anti-inflammatory cytokines. For this purpose we prepared anti-oxidant and phospholipid containing lipid-based formulations. This is the first time we successfully made a combination α-tocopherol and phosphatidyserine in lipid-based formulations for inflammatory treatment. The anti-oxidative and anti-inflammatory property as well as hydrophobic nature of the particles will serve as potential drug carrier at the site of inflammation to induce anti-inflammatory response. This chapter presents background information of this unique approach in this research.

CHAPTER 1

1.1 Macrophage in inflammatory process

Monocytes and macrophages resident in peripheral tissues play an important role in inflammatory process, by assisting the defense system of body against foreign particles. Monocytes are derived from myeloid progenitor cells in bone marrow, which then enter into the peripheral tissue from circulation and named as resident tissue macrophages(Fig. 1) [2-4]. Resident macrophages in different tissues adapt to their local microenvironment and exhibit diverse functional and morphological phenotypes.

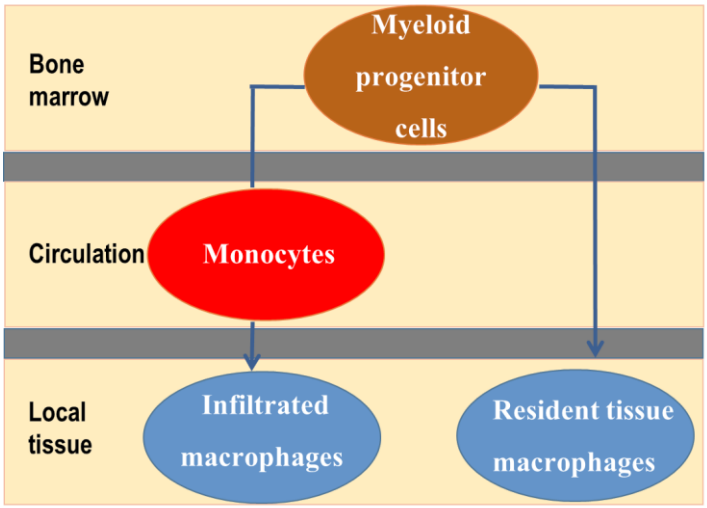


Fig.1. Macrophage lineages

1.1.1 Diversity of macrophages

The macrophages have diverse functions, such as protecting against microorganisms, exerting cytotoxic activity against tumor cells, clearing apoptotic cells, and tissue remodeling. According to the specific tissues that macrophages reside in, they are given unique names such as microglia (central nervous system, CNS), osteoclasts (bone), alveolar

macrophages (lung), Langerhans cells (skin), Kupffer cells (liver), *etc*. The specialized roles of tissue macrophages are directed by local microenvironments [5].Tissue resident macrophages undergo activation in response to various inflammatory and immune stimuli, and then promote further monocyte infiltration to enhance the resident macrophage population, or stimulate an altered phenotype [6].

1.1.2 Macrophage heterogeneity

Macrophages are phenotypically polarized by their microenvironments to mount specific functional programs. Two distinct states of polarized macrophages have been categorized; the classically activated M1 and the alternatively activated M2 phenotype. M1 and M2 macrophages have opposing functional characteristics and have distinct cytokine secretions and surface markers [7-11].

	Classical activated M1	Alternative activated M2
Function	Defense inflammation	Resolve inflammation
Secretions	Pro-inflammatory	Anti-inflammatory
Activation	Endotoxin, Interferon	Interleukins (e.g. IL-4, IL-10 and IL-13)

Fig.2. Heterogeneity of macrophages

The classically activated macrophages (M1) are usually modeled by *in vitro* stimulation with IFN-γ/or LPS stimulation, and are associated with increased microbicidal activity, antigen-presenting functions, and tissue destructive functions. They express elevated genes that are associated with increased pro-inflammatory features such as MHC class II molecules, nitric oxide synthase 2 (NOS2), IL-12, TNFα, IL-6, *etc*. The alternatively activated macrophages (M2) are usually modeled by *in vitro* stimulation with IL-4 or IL-13(Fig.2), and are associated with the anti-inflammatory functions linked to wound healing, fibrosis, tissue repair, allergy, parasite infection, and tumor progression. Elevated anti-inflammatory gene expression was observed in M2 including IL-10, TGFβ1, arginase 1 (Arg 1), and mannose receptor 1 (MR), *etc*. [10, 12].

1.1.3 Macrophages and resolution of inflammation

Macrophages are the 'big eaters' of the immune system. They engulf apoptotic cells and pathogens and produce immune effector molecules. Upon tissue damage or infection, monocytes are rapidly recruited to the tissue, where they differentiate into tissue macrophages. Through their ability to clear apoptotic cell and pathogens and instructing other immune cells, they play a central role in protecting the host by resolving the inflammatory response.

1.1.3.1 Recognition of apoptotic cell by macrophages

Apoptosis is the process of programmed cell death that occur in multicellular organisms. Characteristic morphological changes occur in cell during the apoptosis. Along with the morphological alteration, the surface of apoptotic bodies also modified which lead to the

eating of the apoptotic cell by the macrophages. The macrophages can recognize the apoptotic cell with the help of "find me" signals. The find me signals produced by some signaling molecule such as fractakline, lysophosphatidycholine (LPC), sphingosine-1-phosphate and nucleotides which bind to the receptors CX3CR1, G2A, SIP-R1/5 and P2Y2 respectively (Fig.3)[13].

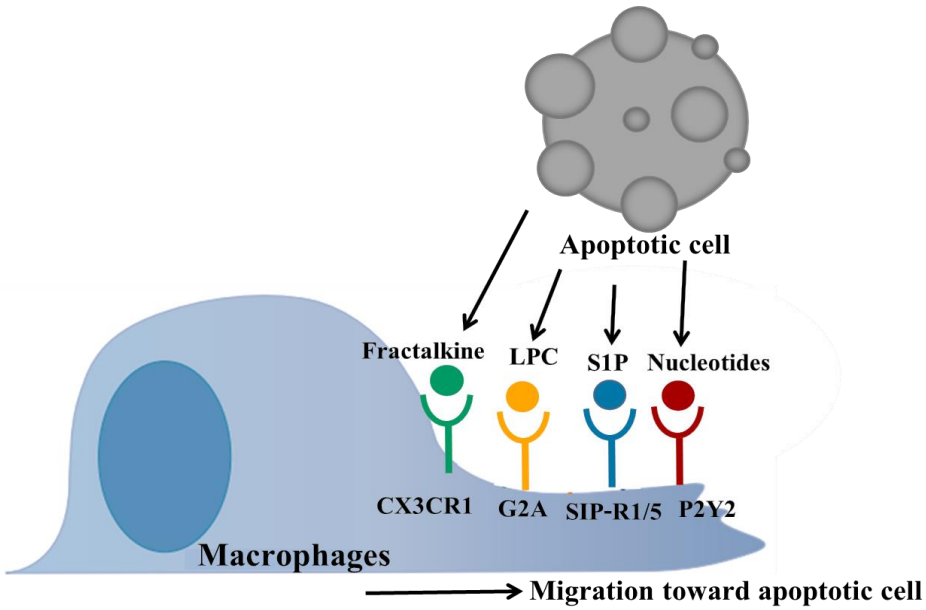


Fig.3. "Find me" signals and their receptors. Apoptotic cells release "find me" signals including fractalkine, LPC, S1P, and nucleotides. These molecules bind their cognate receptors (CX3CR1, G2A, S1P-R1/5, and P2Y2, respectively) present on the macrophage surface. "Find me" signal recognition by the macrophage stimulates migration toward the dying target.

1.1.3.2 Engulfment of apoptotic cell by macrophages

The cell membrane of eukaryotic cell consists of various phospholipid such amino phospholipid, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phophatidylserine (PS) and sphingomyelin (SPM) [13]. Phosphatidylserine is a negative charged phospholipid, present in the inner leaflet of lipid bilayer of the plasma membrane. During apoptosis it flips to the outer leaflet of plasma membrane as a marker of the apoptotic cell. The exposed PS on the cell surface serves as an "eat me" signal to induce phagocytosis of the dead cells by the macrophages.

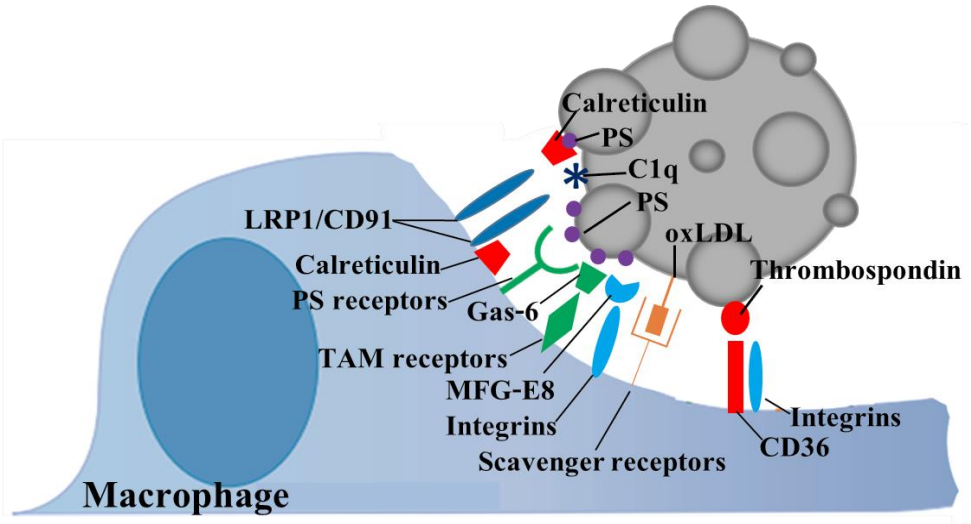


Fig.4. Apoptotic cell "eat me" signals and phagocytic receptors. As apoptotic cells undergo programmed cell death, they begin to expose "eat me" signals on their surfaces. "Eat me" signals are recognized by phagocytic engulfment receptors of PS either directly or indirectly via bridging molecule MFG-E8 [13].

These various types of receptors and bridging molecule help in the process of apoptotic cell recognition and engulfment (Fig.4). MFG-E8 is one of the secreted protein, which can bind to phosphatidylserine on the surface of apoptotic cell promoting the engulfment of apoptotic cells via its receptor integrin. MFG-E8 secreted from phagocyte and has RGD motif which help in association of integrin. It binds with PS with on apoptotic cell through its C1 and C2 domains (Fig.5).

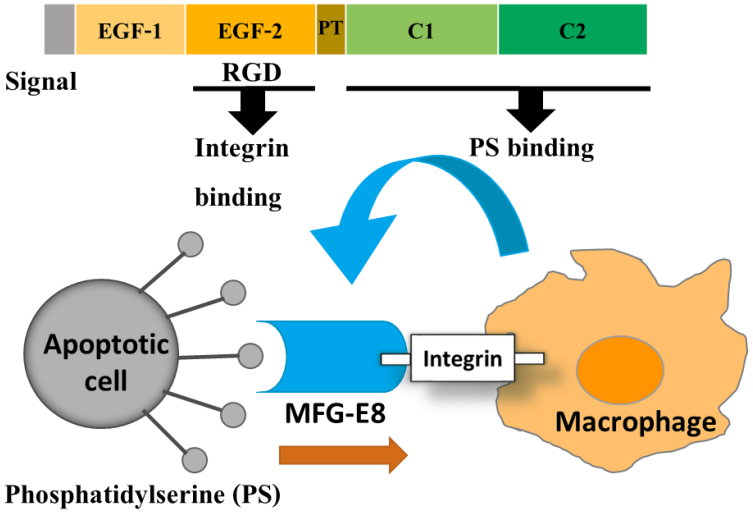


Fig.5. Binding of macrophages to phosphatidylserine

Once the PS has been recognized by macrophage, it leads to polarization of macrophages phenotype. After phagocytic clearance of apoptotic cells, macrophages actively reduce inflammation by decreasing the production of pro-inflammatory cytokines [14-16].

1.2 Drugs used for inflammatory diseases

The most commonly used anti-inflammatory drugs in recent years include glucocorticoids, non-steroidal anti-inflammatory drugs and antibiotics. Though the known anti-inflammatory drugs reduce inflammation, their chronic use is often associated with debilitating side effects [17-20]. In recent years, many research have been done to find the appropriate drugs for reducing the severity of inflammatory diseases. To date no drugs have shown definite beneficial effects in the clinic against chronic inflammatory diseases despite showing good effects in animal models and *in vitro*. The cause of clinical failure could be due to the very narrow therapeutic effects.

1.3 Antioxidant and inflammation

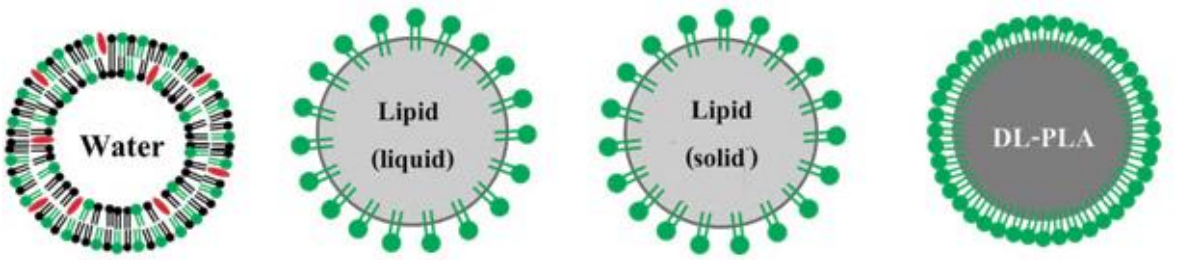
Antioxidants is a promising molecules with anti-inflammatory effects on activated macrophages and microglia [21-24]. In recent years, various antioxidants have undergone preclinical testing that have demonstrated their suppressive effects in inflammatory disease models. Antioxidants induce their anti-inflammatory effects by neutralizing ROS produced by activated macrophages and microglia [25-27]. The antioxidants α-tocopherol and *N*-acetylcysteine are reported to exhibit anti-inflammatory effects in activated macrophages [22]. Idebenone is a synthetic compound that have shown to act on the mitochondria as an antioxidant by inhibiting lipid peroxidation and suppressing non-respiratory oxygen consumption [28, 29].

1.4 Phosphatidylserine and inflammation

Recently, phosphatidylserine (PS) was reported to have anti-inflammatory effects in activated macrophages, microglia and myeloid dendritic cells [16, 30, 31]. PS is a phospholipid found in the inner layer of the cell membrane [32] but during apoptosis it becomes exposed on the cell surface [33-35]. Exposed PS binds directly with macrophage receptors or indirectly *via* bridging molecules such as milk fat globule-EGF factor 8 (MFG-E8), and enhances phagocytosis of apoptotic cells [13, 36, 37]. After phagocytic clearance of apoptotic cells, macrophages actively reduce inflammation by decreasing their secretion of pro-inflammatory mediators [14, 16, 31].

1.5 Lipid-based therapy targeting macrophage activity

In recent years, the use of various lipid-based particles in the area of medical biology, including both diagnostics and therapy, have gained remarkable attention. Design principles of these particles, including nano-emulsions, dendrimers, liposomes, magnetic particles, and lipid nano and micro particles, are primarily based on unique assemblies of synthetic, natural, or biological components. Lipid based particles include Polymeric lipid particles, solid lipid nanoparticles (SLN), nanostructured lipid carriers, lipid drug conjugates and are colloidal drug carrier systems (Fig.6) [38-40]. They are very much like nanoemulsions, differing in lipid nature. The liquid lipid used in emulsions is replaced by a lipid solid at room temperature in SLN including high-melting point lipids [39, 41].



Liposome
(lipid bilayer enclosing an aqueous core)

Nanoemulsion
(lipid monolayer enclosing a liquid lipid core)

Lipid nanoparticle
(lipid monolayer enclosing a solid lipid core)

Polymeric lipid particle
(lipid monolayer enclosing a polymer core)

Fig.6 Structure of lipid-based vehicle systems

However, the potential success of these particles in the clinic relies on consideration of important parameters such as physical properties, drug loading efficiencies, drug release potential, and, most importantly, minimum toxicity of the carrier itself. Among these, lipid-based particles bear the advantage of being the least toxic for in vivo applications, and significant progress has been made in the area of drug delivery. Because of the phagocytic activity of macrophages, lipid based particles can be used as efficient vehicles for the intracellular delivery of drugs. After the uptake by macrophage, the phospholipid bilayers are disrupted by phospholipase and the release of the encapsulated drug lead to an intracellular accumulation of the drug [42].

1.6 Background of this research

Inflammation participates in host defenses against infectious agents and injury, but play a critical role for the development of many complex diseases and disorders including autoimmune diseases, neurodegenerative diseases, inflammatory bowel diseases, and cardiovascular diseases. Interactions of cells in the innate immune system, adaptive immune system, and inflammatory mediators harmonize the aspects of the acute and chronic inflammation. A coordinated series of common effector mechanisms of inflammation contribute to tissue injury, oxidative stress, tissue remodeling, angiogenesis, and fibrosis. Recruitment of blood leukocytes such as neutrophils, monocytes and macrophages characterize the initiation of this diseases. Among these cells, macrophage plays the vital role for the progression of the diseases by secreting various inflammatory cytokines, reactive oxygen species and reactive nitrogen species.

In neurological disorders such as in ischemic stroke, the reperfusion after ischemia initiates an inflammatory response within a few hours [43] and induces cerebral injury by activating microglia [44], a resident macrophage in the brain. Upon activation, microglia produce multiple pro-inflammatory mediators, including cytokines, reactive oxygen species (ROS), nitric oxide (NO) and prostaglandin E_2 [14, 45-47]. These pro-inflammatory mediators damage both neural cells and the blood brain barrier (BBB). Through the collapsed BBB, macrophages from the blood migrate into the brain tissue and these further exacerbate inflammation, contributing to the propagation of brain injury [48-50].

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterized by itching and redness on the dorsal area of the skin. In AD, macrophages are known to accumulate in acutely and chronically inflamed skin. During the early inflammatory phase,

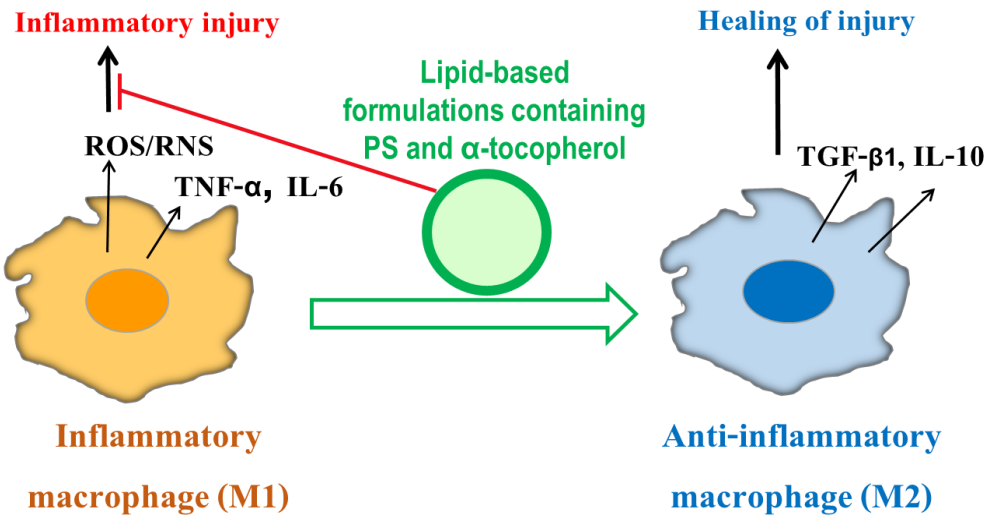
macrophages exert pro-inflammatory functions like antigen-presenting phagocytosis and the production of inflammatory cytokines and growth factors that facilitate the resolution of inflammation. However, persistence of pro-inflammatory activity and altered phenotype of macrophages result in the development of chronic inflammatory phase of AD[51].

Ulcerative colitis (UC) is an inflammatory bowel disease [52], involving the mucosa and sub mucosa of the colon characterized by contiguous inflammation of the colonic lamina propria with subsequent injury and disruption of the mucosal barrier. Multiple factors such as over production of pro-inflammatory mediators including reactive oxygen species (ROS) [53, 54], nitric oxide, cytokines, arachidonate metabolites, and infiltration of activated macrophages in the lamina propria have been implicated in the pathogenesis of ulcerative colitis [55-57]. The infiltrated macrophages secrete many pro-inflammatory cytokines such as IL-6, TNF-α [58], nitric oxide(NO) [57] and generate excess amounts of ROS [59] which exceeds the intestinal defense system, leading to intestinal injury in ulcerative colitis[60]

Thus, the complications of the inflammatory diseases intimately related with inflammatory response produced in macrophages by different signaling pathways. Mastery of the inflammatory response should aid the development of novel strategies to predict disease susceptibility, target therapies, and new approaches to the prevention and treatment of inflammatory diseases.

1.7 Overview of this thesis

Through the mechanism described above, lipid-based particles such as liposome and polymeric lipid particle containing PS might change the inflammatory phenotype of macrophages into anti-inflammatory by binding with PS through bridging molecule MFG-E8 and integrin. Addition of α-tocopherol into the particles will further reduce the inflammatory response of macrophages by neutralization of ROS. The combination of both antioxidants and PS will be able to synergistically reduce the secretion of inflammatory cytokines from the activated macrophages by changing the functional phenotype of macrophages.



To achieve this target, we designed a lipid based formulations containing α-tocopherol and PS and verified their inflammatory response *in vitro* and *in vivo*. Since α-tocopherol is a lipophilic vitamin, it can be loaded on the liposomal membrane as well as core of the polymeric lipid particle. We found that both α-tocopherol and PS containing liposomes and polymeric lipid particles synergistically altered the phenotype of macrophages into anti-

inflammatory state and then inhibit the production of ROS and pro-inflammatory cytokines from the activate macrophages. The content of this thesis is organized as follows.

In chapter 2, I reported the preparation of anti-inflammatory liposomes, containing both phosphatidylserine and α-tocopherol and investigated their effects on anti-inflammatory response of hypoxia activated microglia, the cells that mediate inflammation associated with brain ischemia.

In chapter 3, I prepared PLA micro particle coated with PS on the outside shell and applied this micro particle in the atopic dermatitis mice model for achieving the modulation of the macrophage phenotype to an anti-inflammatory state.

In chapter 4, I focused on decreasing the oxidative stress and inflammatory response produced by activated macrophages using a combination of anti-oxidants and anti-inflammatory molecule. To achieve this target, we designed a biodegradable polymer-lipid hybrid nanoparticle (PLNP) which comprising PLA as polymer cores and PS-polyvinyl alcohol (PVA) as outer shell, exhibit a complementary characteristics of both polymeric nanoparticles and liposomes, particularly in terms of their physical stability and biocompatibility.

In chapter 5, I summarized the findings and discussions described in this thesis. Then, I described the perspectives of the research.

1.8 References

[1] Mills C. M1 and M2 macrophages: oracles of health and disease. Critical Reviews™ in Immunology 2012;32.

[2] van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. The Journal of experimental medicine 1968;128:415-35.

[3] Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71-4.

[4] Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA, Ravasi T. The mononuclear phagocyte system revisited. Journal of leukocyte biology 2002;72:621-7.

[5] Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nature Reviews Immunology 2005;5:953-64.

[6] Gordon S. Alternative activation of macrophages. Nature reviews immunology 2003;3:23-35.

[7] Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. Journal of Clinical Investigation 2007;117:175.

[8] Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. Diabetes 2008;57:3239-46.

[9] Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annual review of immunology 2009;27:451-83.

[10] Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature immunology 2010;11:889-96.

[11] Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science 2010;327:656-61.

[12] Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nature reviews immunology 2011;11:750-61.

[13] Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. Cold Spring Harbor perspectives in biology 2013;5:a008748.

[14] Huynh M-LN, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. The Journal of clinical investigation 2002;109:41-50.

[15] Otsuka M, Goto K, Tsuchiya S, Aramaki Y. Phosphatidylserine-Specific Receptor Contributes to TGF-. BETA. Production in Macrophages through a MAP Kinase, ERK. Biological and Pharmaceutical Bulletin 2005;28:1707-10.

[16] Otsuka M, Tsuchiya S, Aramaki Y. Involvement of ERK, a MAP kinase, in the production of TGF-β by macrophages treated with liposomes composed of phosphatidylserine. Biochemical and biophysical research communications 2004;324:1400-5.

[17] Slatore CG, Tilles SA. Sulfonamide hypersensitivity. Immunology and allergy clinics of North America 2004;24:477-90.

[18] Ciriaco M, Ventrice P, Russo G, Scicchitano M, Mazzitello G, Scicchitano F, et al. Corticosteroid-related central nervous system side effects. Journal of pharmacology & pharmacotherapeutics 2013;4:S94.

[19] Beg S, Swain S, Hasan H, Barkat MA, Hussain MS. Systematic review of herbals as potential anti-inflammatory agents: Recent advances, current clinical status and future perspectives. Pharmacognosy reviews 2011;5:120.

[20] Grill MF, Maganti RK. Neurotoxic effects associated with antibiotic use: management considerations. British journal of clinical pharmacology 2011;72:381-93.

[21] Heppner FL, Roth K, Nitsch R, Hailer NP. Vitamin E induces ramification and downregulation of adhesion molecules in cultured microglial cells. Glia 1998;22:180-8.

[22] Bellezzo JM, Leingang KA, Bulla GA, Britton RS, Bacon BR, Fox ES. Modulation of lipopolysaccharide-mediated activation in rat Kupffer cells by antioxidants. Journal of Laboratory and Clinical Medicine 1998;131:36-44.

[23] Numakawa Y, Numakawa T, Matsumoto T, Yagasaki Y, Kumamaru E, Kunugi H, et al. Vitamin E protected cultured cortical neurons from oxidative stress-induced cell death through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Journal of neurochemistry 2006;97:1191-202.

[24] Sinha J, Das N, Basu M. Liposomal antioxidants in combating ischemia-reperfusion injury in rat brain. Biomedicine & pharmacotherapy 2001;55:264-71.

[25] Devaraj S, Li D, Jialal I. The effects of alpha tocopherol supplementation on monocyte function. Decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. Journal of Clinical Investigation 1996;98:756.

[26] Shirley R, Ord EN, Work LM. Oxidative stress and the use of antioxidants in stroke. Antioxidants 2014;3:472-501.

[27] Love S. Oxidative stress in brain ischemia. Brain Pathology 1999;9:119-31.

[28] SUGIYAMA Y, FUJITA T, MATSUMOTO M, OKAMOTO K, IMADA I. Effects of idebenone (CV-2619) and its metabolites on respiratory activity and lipid peroxidation in brain mitochondria from rats and dogs. Journal of pharmacobio-dynamics 1985;8:1006-17.

[29] Nagy IZ-. Chemistry, toxicology, pharmacology and pharmacokinetics of idebenone: a review. Archives of gerontology and geriatrics 1990;11:177-86.

[30] Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, et al. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. Journal of Clinical Investigation 2002;109:661-70.

[31] Zhang J, Fujii S, Wu Z, Hashioka S, Tanaka Y, Shiratsuchi A, et al. Involvement of COX-1 and up-regulated prostaglandin E synthases in phosphatidylserine liposome-induced prostaglandin E 2 production by microglia. Journal of neuroimmunology 2006;172:112-20.

[32] Krieger M. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annual review of biochemistry 1994;63:601-37.

[33] Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 1997;89:1121-32.

[34] Krahling S, Callahan MK, Williamson P, Schlegel RA. Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages. Cell death and differentiation 1999;6:183-9.

[35] Ravichandran KS. "Recruitment signals" from apoptotic cells: invitation to a quiet meal. Cell 2003;113:817-20.

[36] Verhoven B, Krahling S, Schlegel RA, Williamson P. Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. Cell death and differentiation 1999;6:262-70.

[37] Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RAB, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 2000;405:85-90.

[38] Mehnert W, Mäder K. Solid lipid nanoparticles: production, characterization and applications. Advanced drug delivery reviews 2001;47:165-96.

[39] Muller RH, Keck CM. Challenges and solutions for the delivery of biotech drugs–a review of drug nanocrystal technology and lipid nanoparticles. Journal of biotechnology 2004;113:151-70.

[40] Castelli F, Puglia C, Sarpietro MG, Rizza L, Bonina F. Characterization of indomethacin-loaded lipid nanoparticles by differential scanning calorimetry. International journal of pharmaceutics 2005;304:231-8.

[41] Manjunath K, Venkateswarlu V. Pharmacokinetics, tissue distribution and bioavailability of clozapine solid lipid nanoparticles after intravenous and intraduodenal administration. Journal of Controlled Release 2005;107:215-28.

[42] Van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. Journal of immunological methods 1996;193:93-9.

[43] Jin R, Yang G, Li G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. Journal of leukocyte biology 2010;87:779-89.

[44] Chamorro Á, Hallenbeck J. The harms and benefits of inflammatory and immune responses in vascular disease. Stroke 2006;37:291-3.

[45] Gürsoy-Özdemir Y, Can A, Dalkara T. Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia. Stroke 2004;35:1449-53.

[46] Huang J, Upadhyay UM, Tamargo RJ. Inflammation in stroke and focal cerebral ischemia. Surgical neurology 2006;66:232-45.

[47] Sanderson TH, Reynolds CA, Kumar R, Przyklenk K, Hüttemann M. Molecular mechanisms of ischemia–reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. Molecular neurobiology 2013;47:9-23.

[48] Pun PB, Lu J, Moochhala S. Involvement of ROS in BBB dysfunction. Free radical research 2009;43:348-64.

[49] del Zoppo GJ, Milner R, Mabuchi T, Hung S, Wang X, Berg GI, et al. Microglial activation and matrix protease generation during focal cerebral ischemia. Stroke 2007;38:646-51.

[50] Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia Potentiate Damage to Blood–Brain Barrier Constituents Improvement by Minocycline In Vivo and In Vitro. Stroke 2006;37:1087-93.

[51] Kasraie S, Werfel T. Role of macrophages in the pathogenesis of atopic dermatitis. Mediators of inflammation 2013;2013.

[52] Geier MS, Butler RN, Howarth GS. Inflammatory bowel disease: current insights into pathogenesis and new therapeutic options; probiotics, prebiotics and synbiotics. International journal of food microbiology 2007;115:1-11.

[53] Araki Y, Andoh A, Fujiyama Y. The free radical scavenger edaravone suppresses experimental dextran sulfate sodium-induced colitis in rats. International journal of molecular medicine 2003;12:125-9.

[54] Bitiren M, Karakilcik AZ, Zerin M, Ozardalı I, Selek S, Nazlıgül Y, et al. Protective effects of selenium and vitamin E combination on experimental colitis in blood plasma and colon of rats. Biological trace element research 2010;136:87-95.

[55] Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology 2004;126:1620-33.

[56] De Hertogh G, Aerssens J, Geboes KP, Geboes K. Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease. World journal of gastroenterology: WJG 2008;14:845.

[57] Abdelouhab K, Rafa H, Toumi R, Bouaziz S, Medjeber O, Touil-Boukoffa C. Mucosal intestinal alteration in experimental colitis correlates with nitric oxide production by peritoneal macrophages: effect of probiotics and prebiotics. Immunopharmacology and immunotoxicology 2012;34:590-7.

[58] Mahida YR. The key role of macrophages in the immunopathogenesis of inflammatory bowel disease. Inflammatory bowel diseases 2000;6:21-33.

[59] Grisham MB, Granger DN. Neutrophil-mediated mucosal injury. Digestive diseases and sciences 1988;33:6S-15S.

[60] Babbs CF. Oxygen radicals in ulcerative colitis. Free Radical Biology and Medicine 1992;13:169-81.

CHAPTER 2

Synergy between phenotypic modulation and ROS neutralization in reduction of inflammatory response of hypoxic microglia by using phosphatidylserine and antioxidant containing liposomes toward brain ischemia therapy

2 Introduction

2.1 Ischemic stroke

Ischemic stroke in the brain is a leading cause of death in industrialized countries [1]. The ischemic stroke triggers four major mechanisms such as break down of ionic homeostasis, excitoxicity [2], oxidative stress [3], and inflammation [4, 5] that lead to cell death. Among the four mechanisms oxidative stress and inflammation play the major role in contributing the neurological disorders such as brain ischemia. Reperfusion after ischemia initiates an inflammatory response within a few hours [6] and induces cerebral injury by activating microglia [7], a resident macrophage in the brain. Upon activation, microglia produce multiple pro-inflammatory mediators, including cytokines, reactive oxygen species (ROS), nitric oxide (NO) and prostaglandin E_2 [5, 8-10].

In recent years, many neuroprotective drugs that show promising results in preclinical testing were ineffective in human trials. However, these disappointing outcome could be a result of the narrow therapeutic effects in the trials [11]. In this research, I will elaborate on therapeutic strategies such as using of anti-inflammatory and anti-oxidant agents and their synergistic effects to attenuate or prevent the progress of ischemic injury.

2.1.1 Oxidative stress in cerebral ischemia

Due to high oxygen consumption after reperfusion and low level of antioxidant capacity, the brain is vulnerable to oxidative stress. High levels of unsaturated fatty acids in the brain also causes vulnerable to lipid oxidation damage [12]. The oxidative stress can occur when the cellular level of antioxidant are inadequate to detoxify the generation of free radicals [12, 13]. Calcium pathway plays a role in ROS production. Energy loss also leads to mitochondria dysfunction causing inadequate handling of ROS in cells [14]. However, more pronounced injury occurs during reperfusion, where large amounts of reactive oxygen and nitrogen species are produced due to the re-introduction of oxygen and therefore oxidation [3].

2.1.1.1 Generation of free radicals and reactive oxygen and nitrogen species sources

Nitric oxide radical, superoxide anions, peroxynitrite, and hydroxyl radicals are the common free radicals found in cellular processes that contain an unpaired electron in their outer electron orbital [12]. Free radical reactions are important in the survival of cells because of their involvement in ATP generation, arachidonic acid metabolism (Fig.1.B), and cytochrome enzyme activity [12]. Hydroxyl radical is formed by decomposing of hydrogen peroxide (H_2O_2) by the Fenton reaction, while peroxynitrite can be generated by the reaction of NO and superoxide anions (Fig. 2). The mitochondrion respiratory chain is a major generator of ROS (Fig. 1). Under physiological conditions, endogenous antioxidants scavenge free radical species produced during metabolism and various cellular activities.

During brain ischemia, mitochondria phosphorylation becomes a major source of increased free radicals production through the respiratory chain [15].

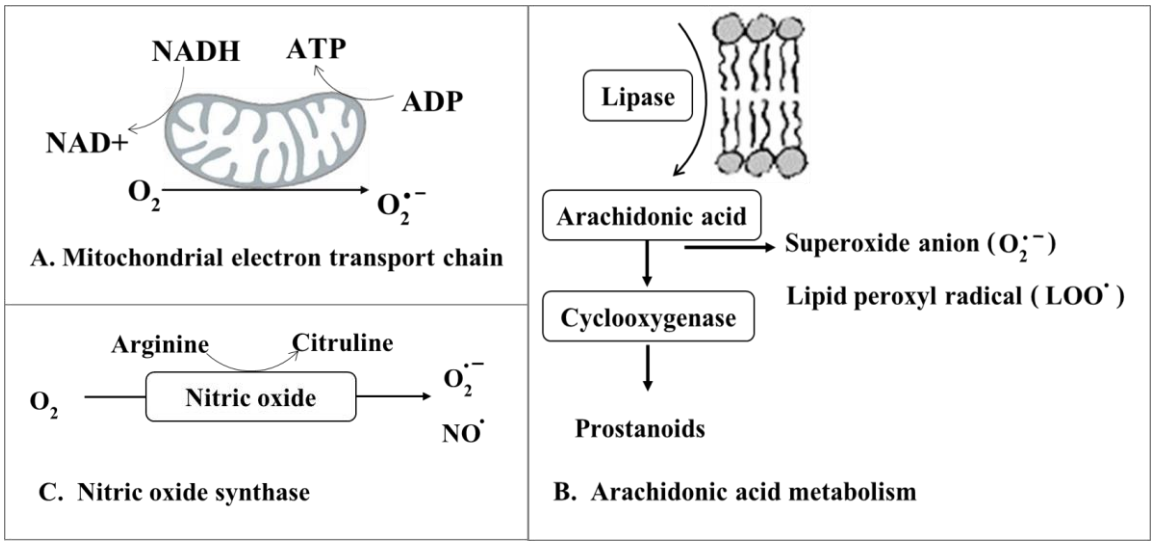


Fig. 1. Sources of oxygen radical species that can contribute to ischemic injury: **A**. mitochondrial electron transport chain; **B**. metabolism of arachidonic acid; and **C**. nitric oxide synthase.

As a result, ROS production excels and the endogenous antioxidants are not able to scavenge the excess ROS. Elevated intracellular calcium level also leads to activation of various enzymatic systems, such as lipases, endonuclease, and various proteases, and elevate the formation of ROS and NO [16] by the inflammatory cells such as macrophages and microglia, but can be expressed in neurons, astrocytes, and endothelial cells in the brain [3, 17] .

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{\bullet} \quad \textbf{(Fenton reaction)}$$

$$NO^{\bullet} + O2^{\bullet -} \longrightarrow ONOO^{\bullet} + NO2^{\bullet -} + {}^{\bullet}OH \quad \textbf{(Peroxynitrite formation)}$$

Fig.2. Formation of hydroxyl radicals by Fenton reaction. Nitric oxide and superoxide anion react to form peroxynitrite.

2.1.1.2 Endogenous antioxidant defense mechanisms

There are various antioxidant defense mechanisms such as superoxide dismutase, catalase, glutathione peroxidase, and other reductants for example glutathione, ascorbate, and α-tocopherol present in the brain. Neuronal death and edema in the brain are reported to increase in superoxide dismutase deficient mice [18]. Glutathione, ascorbate (Vitamin C), and α-tocopherol (Vitamin E) also play important roles in protecting the brain from cerebral ischemia [19].

2.1.2 Inflammatory process in cerebral ischemia

Microglia, the residential macrophage of brain, plays a major role in the initiation of inflammatory process during the onset of cerebral ischemia and subsequent tissue damage in both acute and chronic neurological diseases. Immediately after ischemia, necrotic cell, oxidative stress, and hypoxia initiate the innate inflammatory response through calcium ion activated enzymatic systems that increase oxygen free radicals and synthesis of various pro-inflammatory mediators [20]. The prominent transcription factors involved in gene expression of pro-inflammatory mediator during ischemia are hypoxia inducible factor 1 [21], nuclear factor kappa B [22], and signal transducer and activator of transcription factor 3 (STAT3) [23]. These transcription factors produce mediators of inflammation from the activated macrophages, which include tumor necrosis factor alpha (TNFα) [24]. Chemokines are also produced to attract the inflammatory cells in the site of damage[25].The macrophages from the blood migrate into the brain tissue and these further exacerbate inflammation, contributing to the propagation of brain injury[5, 8, 9].

2.1.2.1 Microglia

The physiological function of neurons is supported by the large number of glial cells [26]. Among these cells, a unique population of neural environment-adapted macrophages comprises more than 12% of the non-neuronal brain cells and is known as microglia [27, 28]. These cells are distributed throughout the central nervous system (CNS), but they vary in density, with fewer microglia in white matter than the grey matter [29, 30].

These cells are derived from a mononuclear phagocyte lineage and possess several morphological features and functions with macrophages, constituting the immune system of brain. Thus, microglia play an essential role in both physiological and pathological conditions [31].

2.1.2.2 Microglial phenotypes

Microglial phenotypes are solely divided in a resting and an activated state. In healthy normal CNS, microglia takes the resting state presenting small cellular body with rod-shaped nuclei and long branching processes. In pathological conditions, microglia turn into an activated state with several morphological alterations (Fig.3) [32, 33]. Cells in the resting state have been considered as quiescent and inactive. Indeed, microglial cells can be detected in neighborhood of micro-damaged site and play regenerative functions by initiating an inflammatory response. When the stimuli are stronger or prolonged, microglial cells undergo a dramatic changes [33]. In recent years, the concept of different states of microglial activation responsible for the differing functional properties are described [35]. This idea became visible from research in non-CNS field to perceive whether macrophages play a harmful or beneficial role after injury and expressed as macrophage polarization.

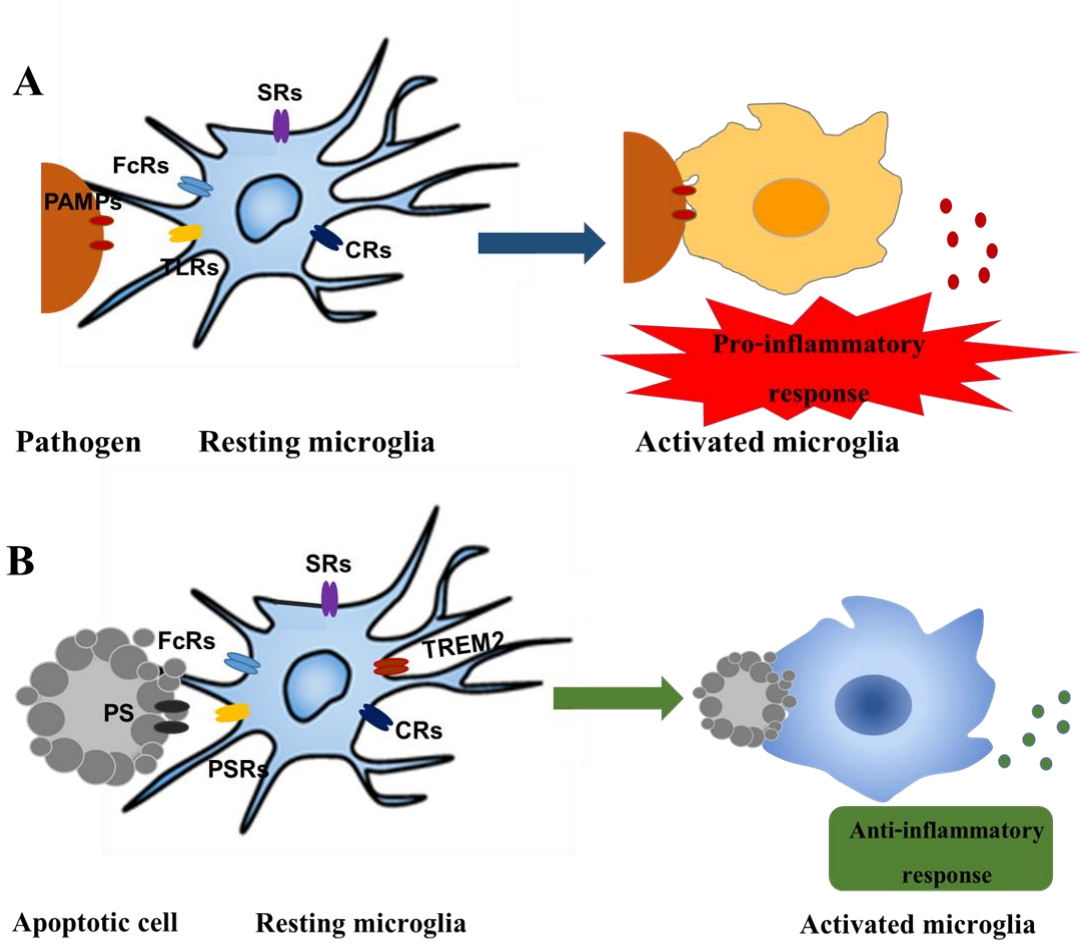


Fig. 3. Phagocytic receptors of microglia and their inflammatory response on activation. **A**. Microglia recognize specific structural patterns (PAMPs) of most microbial pathogens via their TLRs, complement receptors (CRs), Fc Receptors (FcRs) or scavanger receptors (SRs), leading to a pro-inflammatory response. **B**. Microglia recognize apoptotic cells through phosphatidylserine residues (PS) expressed on the membrane of the apoptotc cells. These residues are recognized by PS receptors, which are supported via additional phagocytic receptors including TREM2. Phagocytosis of apoptotic cells induces the release of anti-inflammatory cytokines [34].

The classical (M1) activation, induced by interferon (IFN)-γ, is characterized by a pro-inflammatory response to eliminate the extracellular pathogens. On the other hand, alternative (M2) activation important for the immune response to parasites and tissue repair

[35, 36]. However, these polarization states (M1 and M2) describe a complex process and belongs a broad spectrum of functional states [37]. Based on these concepts, microglia is quite simple in the case of classical activation (M1) as well as alternative activation (M2).

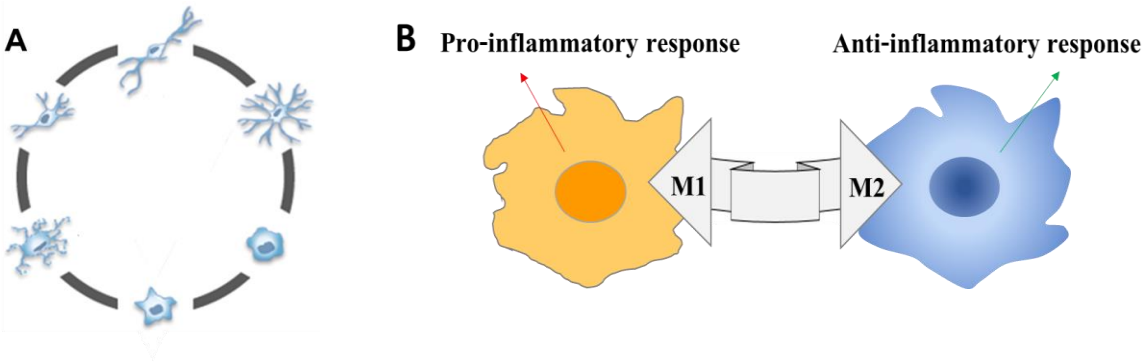


Fig.4. Microglial phenotypic diversity**.** **A.** Microglial cells show a great phenotypic heterogeneity and plasticity being capable of quickly adaption to achieve an appropriated effector response for each challenge to CNS. **B.** Recently, it was proposed the concept of different states of activation, ranging from "classical" activation (or M1) to "alternative" activation (or M2), which represent the extremes of a spectrum of functional states.

However, the steady-state of microglia is more challenging and the determinants of this state might be very distinct from those of macrophages. It is not known that the deactivation of microglia results in a state functionally similar to that of resting state. But, some research have been reported to support the association between distinct phenotypes and pathology [36]. Therefore, macrophages and microglia should recognize different signals from their microenvironment that lead to specialized activation programs [38] and induce divergent effects in response to CNS injury [39]. Thus, depending on their differentiation state, microglia affects the cellular function with either a deleterious or beneficial outcome in the process of tissue repair [40]. The microglial phenotypes based on classic morphologic studies is now replaced by a definition of functional states (Fig.4). This new definition

differentiate the activation states of microglia to achieve appropriated effector responses to overcome the challenges of CNS [41]. In recent years, discovery of microglial involvement in neurogenesis and post lesion undertakes functionally adapted microglial phenotypes [42].

2.1.2.3 Role of microglia in neuroinflammation

Microglial cells are the source of inflammatory mediators that may either have a neurotoxic or neuprotective effect (Fig.5). In physiological conditions, microglia contributes to growth, functional maintenance and phenotypic development of neuronal cells as well as proliferation and growth of glial cells by producing neurotrophic factors [43, 44]. Activation of microglia upon any stimulation/injury has been considered as a detrimental effect. This detrimental event came from considering the inflammatory and cytotoxic phenotype of LPS-stimulated microglia in cell-cultures [33, 45]. In recent studies it suggest that under pathological conditions, microglia exert neuroprotective functions through the production of neurotrophic molecules and by clearance of apoptotic cells [46].

Indeed, microglia protect neurons in damaged brain by secreting anti-inflammatory cytokines and growth factors such as IL-10, TGF-β [47]. TGF-β appears to act as an autocrine mediator by regulating negatively inflammatory and immune regulatory functions of activated microglia, such as suppression of cytokine and ROS production [48]. Neuroprotection by microglia also involves their ability to uptake excitotoxic glutamate by expressing glutamate transporter 1 [44].

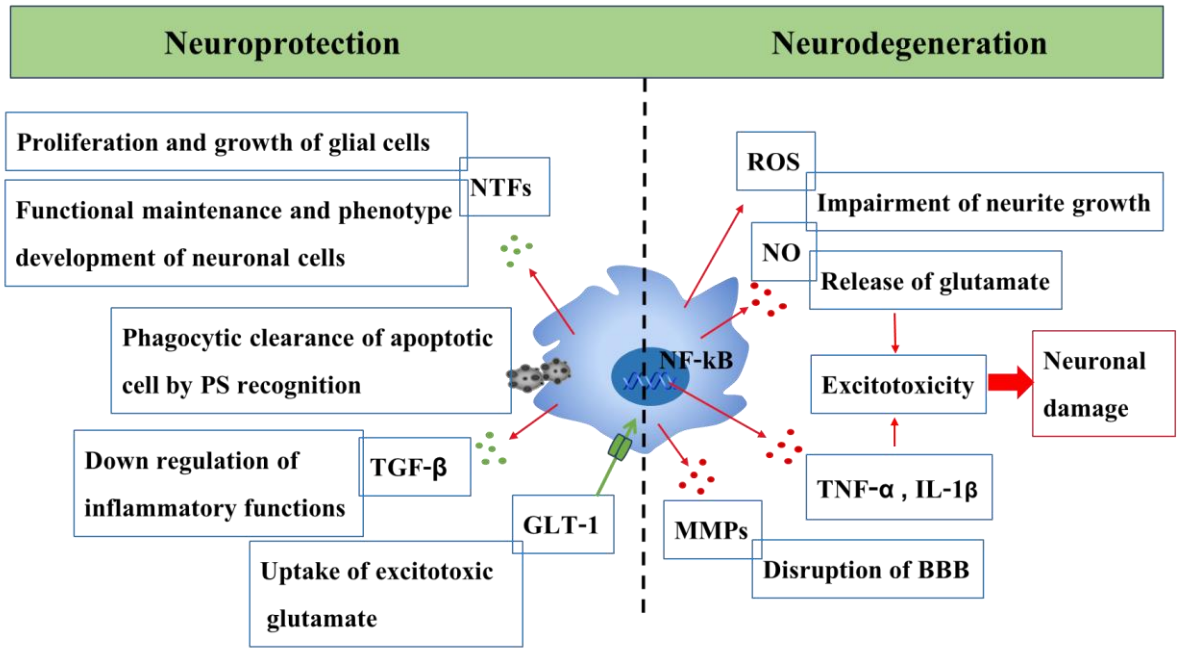


Fig. 5. Neuro-inflammatory mediators produced in neuroprotection and neurodegeneration. Microglia exert neuroprotective functions through the production of neurotrophic factors (NTFs) and by the clearance of cell debris, during physiological and under pathological conditions. Also, they express a glutamate transporter (GLT-1) responsible for the uptake of excitotoxic glutamate. In certain conditions, activation of microglia can exert neurotoxic functions by producing nitric oxide (NO) and reactive oxygen species (ROS) that intertfere with neurite growth and contribute to the release of glutamate. Pro-inflammatory gene expression by nuclear factor (NF)-κB result in pro-inflammatory cytokines production, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Release of these mediators contributes to excitotoxicity resulting in neuronal death. In addition, matrix metalloproteinase (MMPs) are released leading to the disruption of the blood-brain barrier.

In fact, microglia contribute to host defense and repair with immune cytokines acting as neurotrophic substances, protecting and promoting the neural development. However, with intense activation cytokines and other mediators released by microglia can be very destructive and maintenance of brain damage [49, 50]. Secretory components include ROS and nitrogen intermediates, chemokines, pro-inflammatory cytokines and excitotoxins, such

as glutamate [51]. TNF-α is a pro-inflammatory cytokines, early and prominently produced by microglia upon activation, considered to have a critical role in neuropathology. Indeed, TNF-α lies at the beginning of a signal cascade that potentiates glutamate neurotoxicity and can stimulate the production of IL-1β, IL-6 and other cytotoxic cytokines [52, 53]. Thus, cytotoxic properties of microglia can be modulated by cytokines themselves. For instance, IFN-γ prime microglia to become activated for producing of reactive nitrogen intermediates and TNF-α, following LPS treatment [54]. NO is the mainly free radical produced by microglia upon activation by several stimulants such as LPS [55]. In co-cultures with neuron, microglia production of ROS lead to the reduction of neural growth [56]. NO also induces glutamate release from neurons that leads to activation and subsequent excitotoxicity [57].

In recent research, LPS model of microglia activation have shown that LPS acts on the specific microglial receptors TLR4 and Mac-1 triggering a signaling pathway that results in pro-inflammatory gene expression mediated by NF-κB activation and neuronal death [58]. Microglia are also a source of MMPs, which expression can be induced by LPS stimulation. These enzymes have been shown to degrade components of the basal lamina, leading to the disruption of the BBB and thus, contributing to the neuroinflammatory response in many neurological diseases [59].Therefore, this suggests that reducing or blocking key inflammatory cytokines can be an important therapeutic target to reduce inflammation and further damage after ischemia.

2.1.3 Drugs targeting for neuroinflammation

In recent years, many research have been done to reduce or stop progress of ischemic damage by reducing neuroinflammation. To date no drugs have shown definite beneficial effects in the clinic despite showing good neuroprotection against ischemic damage in animal models and *in vitro* [60]. The cause of clinical failure could be due to the very narrow therapeutic effects in these studies. Moreover, therapeutic compounds need to cross the blood-brain barrier and reach to the site of injury. Since oxidative stress and inflammation play a predominant role in the progression of ischemic neurodegeneration, it is suggested that reducing inflammation and oxidative stress can prevent further ischemic damage. Moreover, interfering with these mechanisms could provide relief for endogenous antioxidant systems by assisting in ROS prevention.

2.1.3.1 Anti-inflammatory drugs

The most commonly used anti-inflammatory drugs in recent years include glucocorticoids, non-steroidal anti-inflammatory drugs and antibiotics. These drugs have also been used to control neuroinflammation in experimental models, and in some instances they have shown direct functional effects on microglia. Their anti-inflammatory actions include inhibiting COX activity and NF-kB/ MAPK p38 signalling in activated macrophages and microglia [61-66]. Though the known anti-inflammatory drugs reduce neuroinflammation, their chronic use is often associated with debilitating side effects [67-70].

2.1.3.2 Antioxidant and inflammation

Antioxidants are other examples of promising molecules with anti-inflammatory effects on activated macrophages and microglia [71-74]. In recent years, various antioxidants have undergone preclinical testing that has demonstrated their suppressive effect on inflammation in neurodegenerative disease models. Antioxidants induce their anti-inflammatory effects by neutralizing ROS produced by activated macrophages and microglia [75-77]. For example, the antioxidants α-tocopherol and *N*-acetylcysteine are reported to exhibit anti-inflammatory effects in activated macrophages [72]. Idebenone is a synthetic compound that have shown to act on the mitochondria as an antioxidant by inhibiting lipid peroxidation and suppressing non-respiratory oxygen consumption [78, 79]. It also improves cerebral energy metabolism in rats during ischemia and protects against neurological deficits [78, 80].While antioxidants have often been suggested for prevention of neurodegenerative diseases, an antioxidant alone would not be sufficient for treatment of these diseases [76]**.**

2.1.3.3 Phosphatidylserine and inflammation

Recently, phosphatidylserine (PS) was reported to have anti-inflammatory effects in activated macrophages, microglia and myeloid dendritic cells [81-83]. PS is a phospholipid normally found in the inner layer of the cell membrane [84] but during apoptosis it becomes exposed on the cell surface [85-87]. Exposed PS binds directly with macrophage receptors or indirectly *via* bridging molecules such as milk fat globule-EGF factor 8 (MFG-E8), and enhances phagocytosis of apoptotic cells [88-90]. After phagocytic clearance of apoptotic cells, macrophages actively reduce inflammation by decreasing their secretion of pro-inflammatory mediators [82, 83, 91]. The clearance of apoptotic cells by macrophages and

microglia through PS-mediated signalling is a key process resulting in anti-inflammatory effects [92].

2.1.4 Liposome to improve inflammatory drug delivery

Liposomes are nano-sized and non-toxic biodegradable vesicles that consist of one or more concentric outer phospholipid bilayers, enclosing an aqueous inner compartment. The structure of liposomes similar to the one of biological membranes that is composed of amphiphilic phospholipids. Liposomes form spontaneously when phospholipids are dispersed in water. Depending on the preparation method and the lipid composition, liposomes may take small unilamellar vesicles (SUV, 50 nm) to multilamellar vesicles (MLV, 1 μm). Liposomes can be loaded with a broad selection of drugs as well as with a variety of biologically active substances [93]. Hydrophilic molecules are trapped in the aqueous liposome interior, whereas lipophilic molecules are incorporated in the outer liposomal membrane. Liposomes can carry a high concentration of the drug that can gradually be released to maintain a therapeutic drug level in the blood or at the local administration site for prolonged periods of times [94]. Drugs or biologically active molecules can be encapsulated in liposomes are protected from enzymatic degradation and inactivation or immunological reaction in the blood stream as well as the degree of non-specific unwanted toxicities [95]. As liposomes can interact with target cells in various ways, they are able to promote the intracellular delivery of drug molecules that in their free form [94].

2.1.5 Liposome-based therapeutic strategy targeting macrophages

Because of the phagocytic activity of macrophages, liposomes can be used as efficient vehicles for the intracellular delivery of drugs. After uptake the phospholipase-mediated disruption of the liposomal phospholipid bilayers and the release of the encapsulated drug lead to an intracellular accumulation of the drug in macrophages [96].

2.1.6 Overview of this chapter

Ischemic injuries involves the four main pathways such as excitotoxicity, ionic imbalance, oxidative stress, and inflammation. Among these, (a)oxidative stress, caused by excess production of reactive oxygen species (ROS) and nitrogen species(NO) and (b) inflammation, involves the production of pro-inflammatory cytokines(TNF-α), play the major role in ischemic injury. In this chapter, I focused on decreasing of the oxidative stress and inflammation by using anti-oxidants and anti-inflammatory molecule. I proposed for the first time, combined treatment with antioxidants and PS for inhibiting microglial activation to reduce inflammation in ischemic brain.

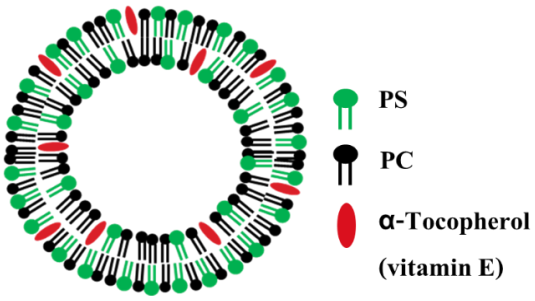


Fig.6. Antioxidant liposome

To achieve this target, I designed a simple antioxidant liposomes (PST-liposomes), containing both PS and α-tocopherol (Fig.6), and verified their suppression of inflammation in hypoxic microglia *in vitro*. These agents might be a good candidate for reducing ischemic injuries since they will assist endogenous anti-inflammatory and anti-oxidative mechanisms in neurological diseases. This can be explained in more precisely by the diagram shown below (Fig.7).

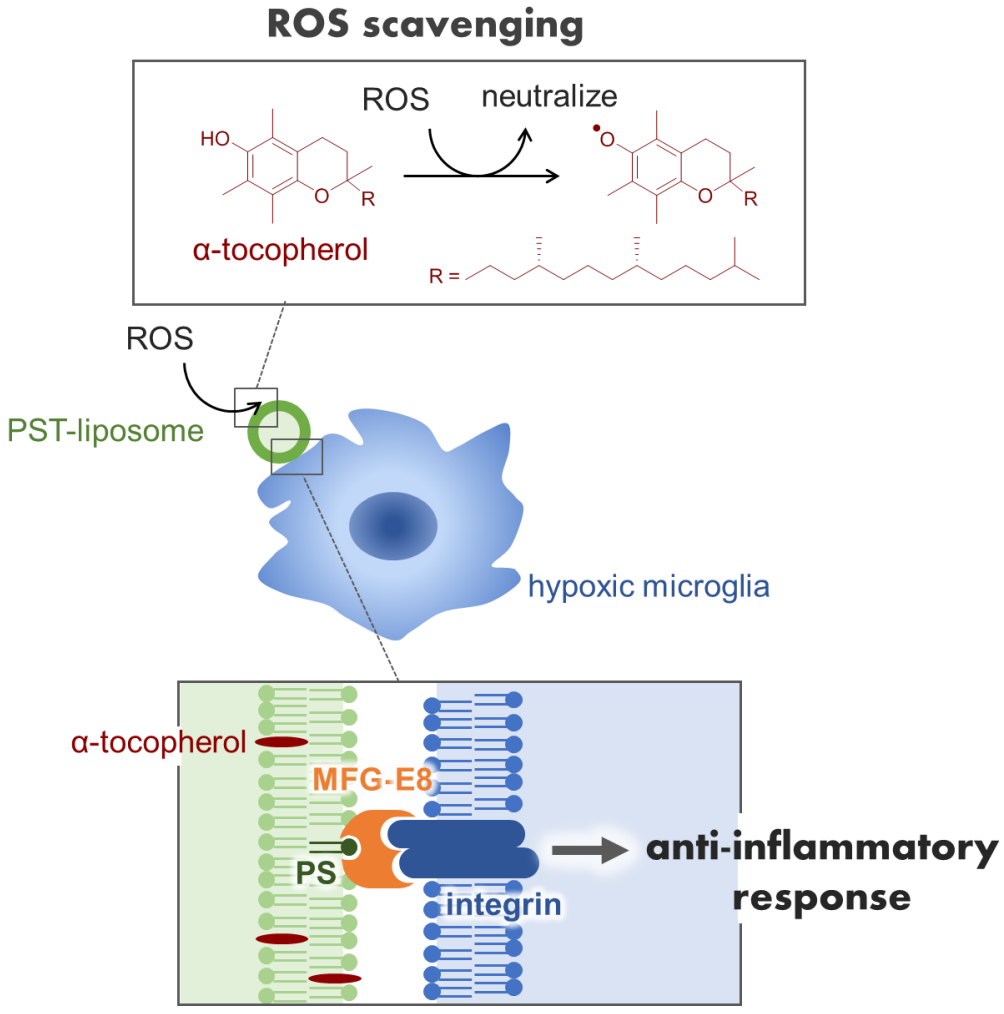


Fig. 7. Expected synergic action of PST-liposome in reduction of inflammatory response of hypoxic microglia.

2.2 Results and Discussion

2.2.1 Characterization of liposomes

We prepared three kinds of liposomes by a standard hydration method. The characteristic values of the liposomes are summarized in Table 1. The average diameter of the PC-, PS- and PST-liposomes were 289, 244 and 200 nm and the ζ-potentials were -2.3, -36.8 and -42.6 mV, respectively. The negative ζ-potentials of the PS- and PST-liposomes reflect the negatively charged PS on the liposome surfaces. Representative dynamic light scattering spectra for the three liposomes are shown in Fig. 8. All the liposomes have similar sizes and relatively narrowly distributed diameters. The somewhat wider size distribution of PC-liposomes, as compared with the others, is likely to result from increased aggregation because of its neutral surface charge.

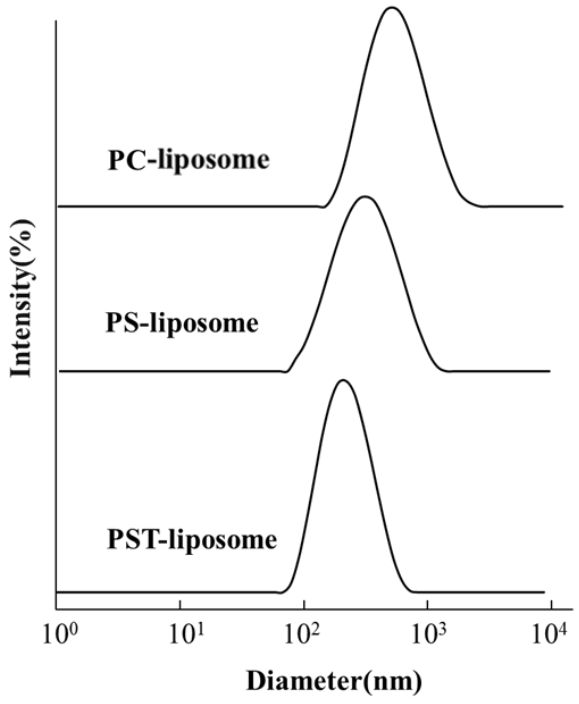


Fig. 8. Size distributions of the prepared liposome.

Table 1. Size, ζ-potential and PdI values of the liposome preparations in PBS.

Name	mole ratio				Size (nm)	ζ-potential (mV)	PdI
	PS	PC	cholesterol	α-tocopherol			
PC	-	0.73	0.27	-	289 ± 5.26	-2.3 ± 0.04	0.57
PS	0.49	0.24	0.27	-	244 ± 0.78	-36.8 ± 0.57	0.23
PST	0.44	0.22	0.27	0.07	200 ± 0.68	-42.6 ± 0.10	0.24

2.2.2 Liposome uptake by microglia

Apoptotic cells and PS-containing liposomes are known to be engulfed by macrophages through endocytosis *via* PS-specific manner [88, 97]. Once the PS is recognized by PS receptors on cell surface or endosome, it engulf by macrophages. Here we investigated the uptake of liposomes by hypoxia-treated microglia with fluorescence microscopy (Fig.9.A) after the fluorescence labelling (DiD labelling) of the liposomes. Red fluorescence was observed in cytosol of the microglia after incubation with PS- and PST-liposomes, indicating that these fluorescently labelled liposomes had been engulfed by the cells. The cellular uptake of the liposomes was also quantitated by flow cytometry (Fig.9.B). The microglia treated with PS- and PST-liposomes showed significantly higher fluorescence than those treated with PC-liposomes and without treatment. Because cellular uptake of PC-liposomes was almost negligible, we concluded that the engulfment of PS- and PST-liposomes by microglia was resulted from PS- mediated endocytosis [98-103]

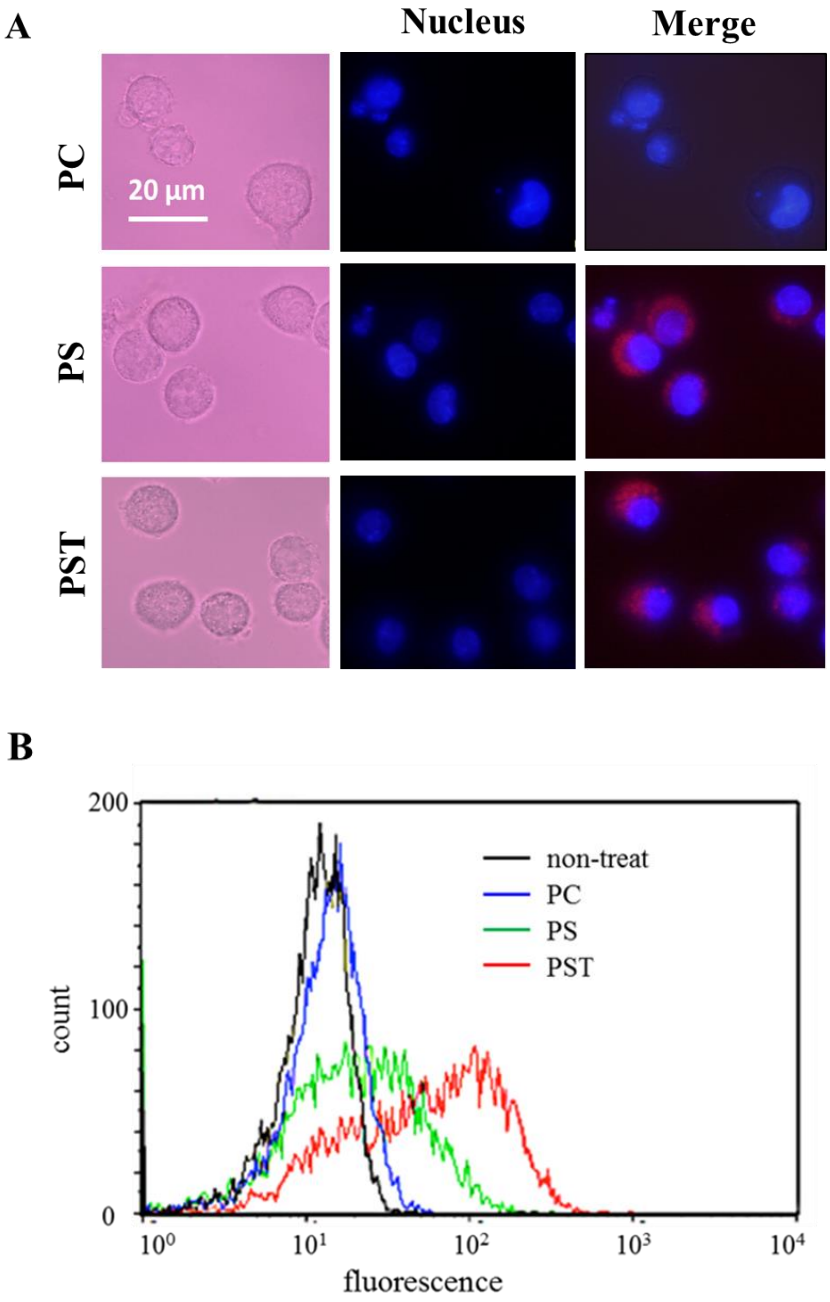


Fig. 9. Uptake of liposomes by microglia : A. Microglial cells were treated with LPS (10 µg/ml) at 37 °C for 1 h and then liposomes (125 µg phospholipid/mL) were added and incubation continued for 3 h at 37 °C. The images were taken with a fluorescence microscope (blue colour is the nucleus, after Hoechst staining, and red indicates liposomes, labelled with DiD); B. Flow cytometry analysis of liposome uptake by microglia.

2.2.3 Improved survival of hypoxia-treated microglia incubated with liposomes

We examined effects of liposome addition on the ability of microglia to resist hypoxia. LPS was added, where indicated, to augment microglial stimulation [104]. As shown in Fig. 10, PC- liposomes did not affect microglial survival, as compared with controls. However, the viability of microglia improved in the presence of PS- and PST-liposomes. Especially, PST-liposomes showed the most significant increase in cell survival. We hypothesized that the superiority of PST-liposomes resulted from a synergy between effects of α-tocopherol and PS, both conferring resistance against hypoxia and LPS stimulation.

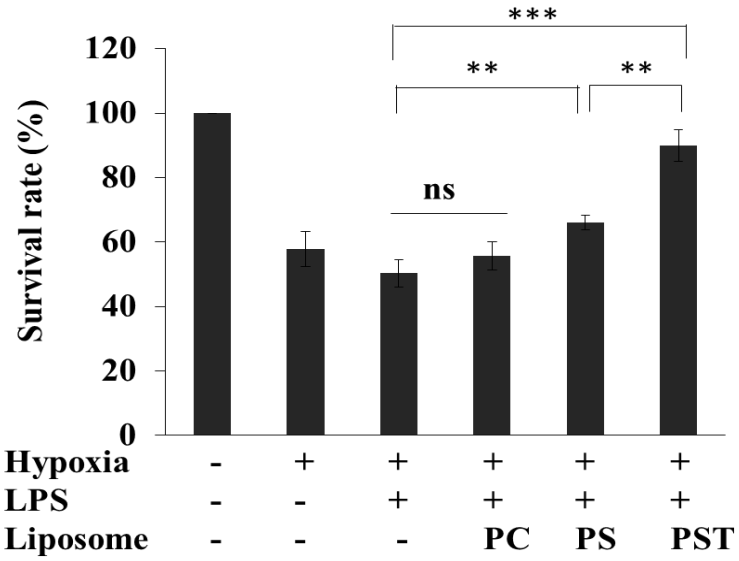


Fig. 10. Microglial cell viability when subjected to hypoxia and LPS stimulation. Microglial cells (5×10^3 cells/well) were seeded in 96 well plates, treated with liposomes (125 μg/ml), where indicated, and incubated for 24 h under hypoxia. Thereafter LPS (10 μg/ml) was added, where indicated, and cells maintained for an additional 6 h under normoxia. Absorbances were measured at 450 nm with the microplate reader. Data are means ± S.D. ns, not significant; **, $p < 0.01$; ***, $p < 0.001$.

2.2.4 Suppression of NO production by hypoxic microglia when treated with anti-inflammatory liposomes

Activated microglia produce NO, which reacts with ROS to further propagate injury in the infarcted areas of the ischemic brain. Thus, suppression of NO production is critical to prevent ischemic injury. Here we examined inhibitory effects of liposomes on NO production from activated microglia under hypoxia. To enhance NO production, the microglia were further stimulated with LPS (10 μg/ml) and IFN-γ (100 U/ml) during the hypoxic treatment.

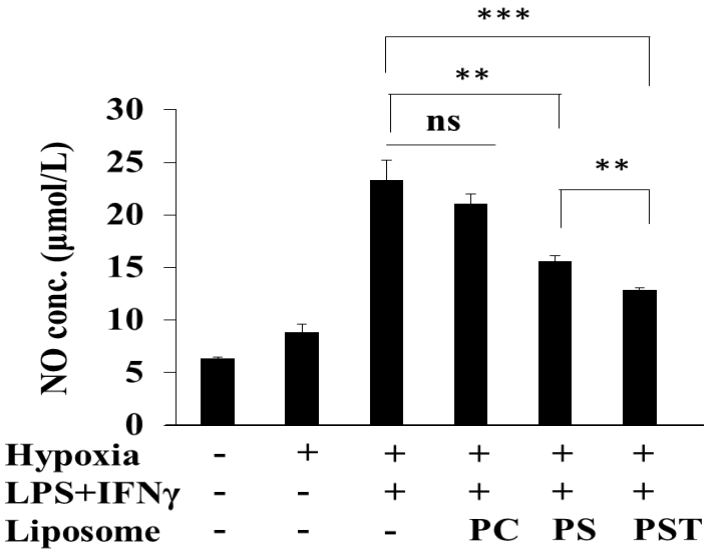


Fig. 11. Effects of liposomes (125 μg/ml) on NO production by microglial cells stimulated with hypoxia (1% O_2) and LPS (10 μg/ml) + IFN-γ (100 U/ml). Data are means ± S.D. ns, not significant; **, p< 0.01; ***, $p < 0.001$.

As shown in Fig. 11, PC-liposomes had no significant effect on NO production. In contrast, significant suppression of NO production was observed with both PS- and PST-liposome treatments, with PST-liposomes producing the greatest effect. Previously, NO suppressing

effects of either PS-liposomes [105] or α-tocopherol [106, 107] in activated peritoneal macrophages were reported. Our findings confirmed that liposomes combining PS and α-tocopherol are even more effective, compared with those having only PS, at reducing NO production from activated microglia.

2.2.5 Reduction of microglial TNF-α production by treatment with anti-inflammatory liposomes

To examine the anti-inflammatory activity of each liposome preparation, we measured production of TNF-α, a typical pro-inflammatory cytokine, from microglia. Because we had found that hypoxic treatment was insufficient to induce TNF-α secretion, we further added LPS.

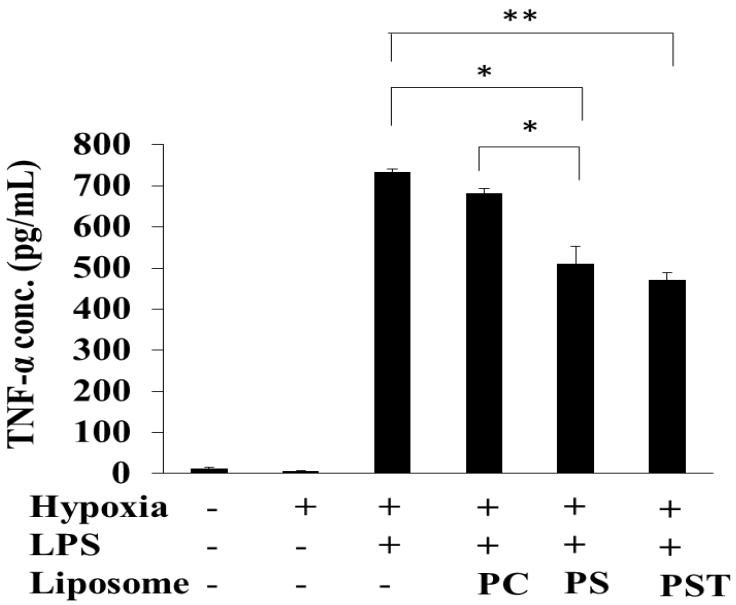


Fig. 12. Effects of liposomes (125 μg/ml) on TNF-α production by microglial cells stimulated with hypoxia (1% O_2) and LPS (10 μg/ml). Data are means ± SD of three separate experiments. ns, not significant; *,$p < 0.05$; **,$p < 0.01$.

In microglia, LPS is known to enhance production of ROS, which mediate pro-inflammatory signalling and amplify TNF-α production [108, 109] through activation of the p38 signalling pathway [108]. As shown in Fig. 12, PS- and PST-liposomes significantly inhibited TNF-α production by microglia treated with both LPS and hypoxia. PS-liposomes have been reported to suppress TNF-α production from microglia [110, 111] and macrophages [112] by affecting various signalling pathways, for example, inhibiting phosphorylation of p38MAPK [111] and downregulating MyD88 [112]. α-tocopherol is also known to decrease TNF-α production, *via* inhibition of 5-lipoxygenase [113]. Our results indicated that PST-liposomes suppressed TNF-α production more effectively than PS liposomes because dominant signalling pathways which are modulated by PS recognition or ROS erasing with α-tocopherol will function independently to reduce TNF-α production.

2.2.6 Effects of antioxidant liposomes on TGF-β1 production by microglia under hypoxia

We next assessed the effects of PST-liposomes on production of the anti-inflammatory cytokine TGF-β1. Inflammation is inhibited by TGF-β1, which acts as a negative regulator of NF-kB activation [114]. As shown in Fig. 13, both PS- and PST-liposomes significantly increased TGF-β1 production from microglia under hypoxia, whereas PC-liposomes were not effective. An additional effect of α-tocopherol on the production of TGF-β1 in hypoxia-treated microglia was not clear in this experiment. In fact, there are no reports showing any effects of α-tocopherol on TGF-β1 production by microglia.

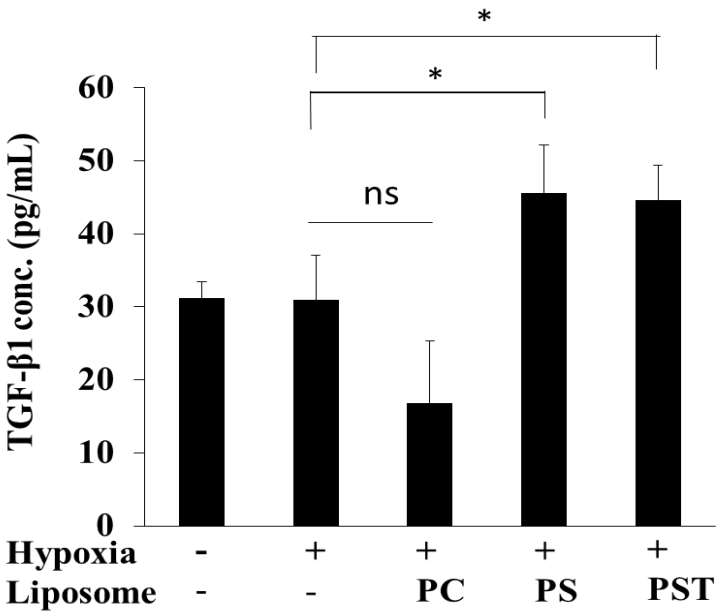


Fig. 13. Effects of liposomes (125 μg/ml) on TGF-β1 production by microglial cells stimulated by hypoxia. Data are means ± SD of three separate experiments. ns, not significant; *, $p<0.05$.

2.3. Conclusion

In this chapter, we reported for the first time the preparation of PST-liposomes, containing both PS and α-tocopherol. These two components showed clear synergistic anti-inflammatory effects on microglia, the cells that mediate inflammation associated with brain ischemia. PST-liposomes significantly increased viability of activated microglia through reducing their production of both NO and inflammatory cytokines. Our data also demonstrated that PST-liposomes are able to alter the inflammatory properties of microglia by inhibiting ROS and inflammatory cytokine production when those cells are stimulated. The synergistic anti-inflammatory effects of PST-liposome indicates that dominant signalling pathways which are modulated either by PS recognition or ROS erasing with α-tocopherol will function independently to cause anti-inflammatory response in microglia. Our PST-liposomes could effectively modulate neuroinflammation and, therefore, have clinical potential for microglial activation-mediated neurological diseases including those involving brain ischemia. Furthermore, interior of PST-liposome is also able to incorporate hydrophilic molecules including cytokines for modulation of microenvironment of neuroinflammation.

2.4 Experiment

2.4.1 Materials

3-sn-Phosphatidyl-L-serine sodium (PS) from bovine brain, L-α-Phosphatidylcholine (PC) from egg yolk and lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111; B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and α-tocopherol was purchased from TCI (Japan). ELISA kits for murine TNF-α, TGF-β1 and IFN-γ were purchased from R and D Systems. A NO assay kit (Griess reagent) was purchased from Dojindo Co. (Japan).

2.4.2 Preparation of liposomes

Multi lamellar liposomes were prepared by the hydration method [115]. Stock solutions were prepared by dissolving cholesterol (20 mg/mL) or phospholipids (PS or PC, 10 mg/mL) in chloroform and α-tocopherol (20 mg/mL) in ethanol.

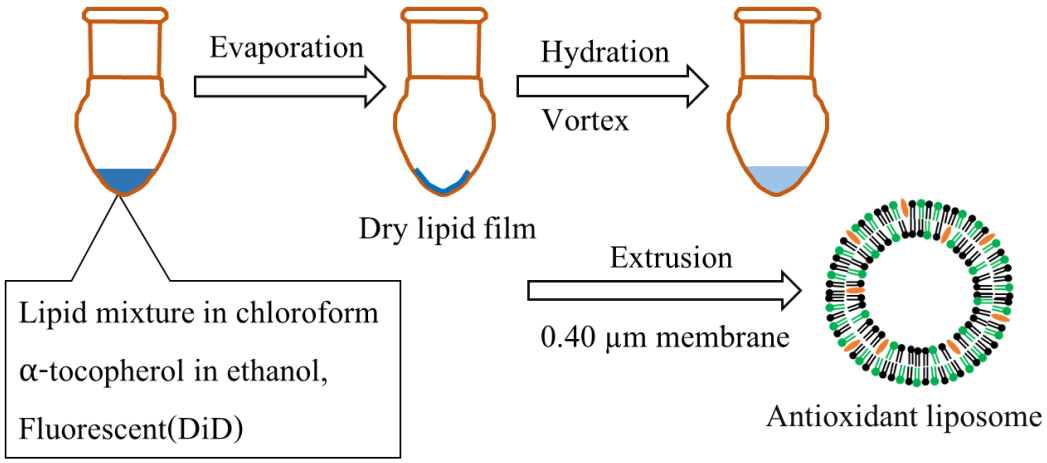


Fig.14. Preparation of liposomes

The stock solutions were added to pear-shaped flasks at the following mole ratios to prepare the indicated liposomes: PS:PC:cholesterol:α-tocopherol = 0.44:0.22:0.27:0.07 (PST-liposomes); PS:PC:cholesterol = 0.49:0.24:0.27 (PS-liposomes); and PC:cholesterol = 0.73:0.27 (PC-liposomes). To study cellular uptake, liposomes were labelled with DiD (Biotium) by adding 1 mol percent to the liposome formulation. Solvents were removed by rotary evaporation and the resulting dried lipid films were hydrated with PBS and vortexed vigorously for 5 min. The liposome suspensions were then extruded with Avanti's mini-extruder using a 0.40 μm polycarbonate membrane.

2.4.3 Measurement of size and ζ-potential

Liposomal size, polydispersity index (PdI) and ζ-potential were measured with a dynamic light-scattering spectrophotometer (Zetasizer Nanoseries, Malvern Instruments, UK) at 25 °C. Results are each reported as the average of three runs.

2.4.4 Cell culture

The immortalized mouse microglial cell line MG6 (Riken Cell Bank, Tsukuba, Japan) was maintained in Dulbecco's Modified Eagle's Medium (Wako) containing 10% foetal bovine serum supplemented with 100 μM β-mercaptoethanol (Gibco), 100 μg/mL insulin (Sigma-Aldrich), 100 μg/mL streptomycin (Sigma-Aldrich) and 100 U/mL of penicillin (Sigma-Aldrich).

2.4.5 Hypoxic exposure and microglial activation

Microglial cells (2 X 10^5 cells) were plated overnight in 35 mm dishes and then cultivated under normoxia (20% O_2 and 5% CO_2) or hypoxia (1% O_2, 5% CO_2 and 94% N_2) at 37 °C for the indicated time periods in a chamber (CO_2/ Multigas Water Jacket Incubator, ASTEC, Japan). For the assay of activation of microglia with and without liposomes, we treated cells with lipopolysaccharide (10 µg/mL) and interferon-γ (100 U/mL) in addition to hypoxic exposure.

2.4.6 Cellular uptake of liposomes

Internalization of DiD-labelled liposomes by microglia was observed by fluorescent microscopy (BZ-8100, Keyence, Japan). To compare the uptake of PS-containing liposomes to that of PC liposomes, flow cytometry analysis (Millipore Guava) was performed.

2.4.7 Cell survival assay

Relative cell viability was measured using the WST-8 cell counting kit (Dojindo Laboratories, Inc., Japan) according to the manufacturer's protocol. Microglial cells (5×10^3 cells/well) were seeded in 100 µL of DMEM containing FBS in 96 well plate. After 24 h of incubation the medium were replaced with FBS free DMEM high and treated with liposomes (125 µg/ml), where indicated, and incubated for 24 h under hypoxia. Thereafter LPS (10 µg/ml) was added, where indicated, and cells maintained for an additional 6 h under normoxia at 37^0C. Thereafter, 10 µL of WST-8 was added to the each well and incubated for 3 h at 37^0C. Conversion WST-8 into formazan by living cells was measured using micro

plate reader (Wallac ARVO.SX 1420 Multilabel counter) at 450 nm absorbance. Total number of living cells were expressed as a percentage relative to untreated control samples.

2.4.8 NO quantification

The accumulation of nitrite (NO_2^-), a stable end product often used as an indicator of NO production by cultured cells, was assayed by the Griess reaction. Microglia ($2X10^4$ cells/mL) were pre-incubated with various liposome preparations (125 µg phospholipid/mL) for 24 h under normoxic conditions. Microglia were further incubated for another 24 h with or without LPS (10 µg/mL) and IFN-γ (100 U/mL) under hypoxia to elicit NO production. After 48 h, cell supernatants were mixed with equal volumes of Griess reagent, incubated at room temperature for 15 min and absorbance was then measured at 540 nm with the microplate reader. Nitrite levels were determined using calibration line prepared by known concentrations of $NaNO_2$ as a standard.

2.4.9 Cytokine detection by ELISA

Microglia were cultured in 35 mm dishes ($2 X 10^5$ cells per dish), treated with liposomes (125 µg phospholipid/mL) and incubated for 24 h under normoxia. The microglia were then further incubated without or with LPS (10 µg/mL) under hypoxia for 24 h. Cell culture supernatants were collected and assayed for TNF-α and TGF-β1 by ELISA. Briefly, the sample supernatants were pipetted onto monoclonal antibody pre-coated microplate for binding with specific cytokine. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokine is added to the wells to sandwich the cytokine immobilized during the first incubation. Following a wash to remove any unbound

antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of cytokine bound in the initial step. The color development is stopped by addition of stop solution and the intensity of the color is measured by micro plate reader at 450 nm absorbance.

2.5 References

[1] Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nature Reviews Neuroscience 2003;4:399-414.

[2] Arundine M, Tymianski M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. Cellular and Molecular Life Sciences 2004;61:657-68.

[3] Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. Journal of Cerebral Blood Flow & Metabolism 2001;21:2-14.

[4] Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. Nature Reviews Neuroscience 2001;2:734-44.

[5] del Zoppo GJ, Milner R, Mabuchi T, Hung S, Wang X, Berg GI, et al. Microglial activation and matrix protease generation during focal cerebral ischemia. Stroke 2007;38:646-51.

[6] Jin R, Yang G, Li G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. Journal of leukocyte biology 2010;87:779-89.

[7] Chamorro Á, Hallenbeck J. The harms and benefits of inflammatory and immune responses in vascular disease. Stroke 2006;37:291-3.

[8] Pun PB, Lu J, Moochhala S. Involvement of ROS in BBB dysfunction. Free radical research 2009;43:348-64.

[9] Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia Potentiate Damage to Blood–Brain Barrier Constituents Improvement by Minocycline In Vivo and In Vitro. Stroke 2006;37:1087-93.

[10] McCarty MF. Down-regulation of microglial activation may represent a practical strategy for combating neurodegenerative disorders. Medical hypotheses 2006;67:251-69.

[11] Rothwell PM, Giles MF, Chandratheva A, Marquardt L, Geraghty O, Redgrave JN, et al. Effect of urgent treatment of transient ischaemic attack and minor stroke on early recurrent stroke (EXPRESS study): a prospective population-based sequential comparison. The Lancet 2007;370:1432-42.

[12] Choi DW, Lobner D, Dugan LL. Glutamate receptor-mediated neuronal death in the ischemic brain. 1998.

[13] Davies KJ. Oxidative stress: the paradox of aerobic life. Biochemical Society Symposia: PORTLAND PRESS-LONDON; 1995. p. 1-32.

[14] Schaller B, Graf R. Cerebral venous infarction: the pathophysiological concept. Cerebrovascular diseases 2004;18:179-88.

[15] Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem j 1973;134:707-16.

[16] Orrenius S, McCabe MJ, Nicotera P. Ca 2+-dependent mechanisms of cytotoxicity and programmed cell death. Toxicology letters 1992;64:357-64.

[17] Nathan C, Xie Q. Regulation of biosynthesis of nitric oxide. J Biol Chem 1994;269.

[18] Fujimura M, Morita-Fujimura Y, Murakami K, Kawase M, Chan PH. Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. Journal of Cerebral Blood Flow & Metabolism 1998;18:1239-47.

[19] Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. Journal of neuroimmunology 2007;184:53-68.

[20] Fandrey J. Hypoxia-inducible gene expression. Respiration physiology 1995;101:1-10.

[21] Ruscher K, Isaev N, Trendelenburg G, Weih M, Iurato L, Meisel A, et al. Induction of hypoxia inducible factor 1 by oxygen glucose deprivation is attenuated by hypoxic preconditioning in rat cultured neurons. Neuroscience letters 1998;254:117-20.

[22] O'Neill LA, Kaltschmidt C. NF-kB: a crucial transcription factor for glial and neuronal cell function. Trends in neurosciences 1997;20:252-8.

[23] Planas AM, Soriano MA, Berruezo M, Justicia C, Estrada A, Pitarch S, et al. Induction of Stat3, a signal transducer and transcription factor, in reactive microglia following transient focal cerebral ischaemia. European Journal of Neuroscience 1996;8:2612-8.

[24] Rothwell NJ, Hopkins SJ. Cytokines and the nervous system II: actions and mechanisms of action. Trends in neurosciences 1995;18:130-6.

[25] Yamasaki Y, Matsuo Y, Matsuura N, Onodera H, Itoyama Y, Kogure K. Transient increase of cytokine-induced neutrophil chemoattractant, a member of the interleukin-8 family, in ischemic brain areas after focal ischemia in rats. Stroke 1995;26:318-23.

[26] Streit WJ. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 2002;40:133-9.

[27] Vilhardt F. Microglia: phagocyte and glia cell. The international journal of biochemistry & cell biology 2005;37:17-21.

[28] Ladeby R, Wirenfeldt M, Garcia-Ovejero D, Fenger C, Dissing-Olesen L, Dalmau I, et al. Microglial cell population dynamics in the injured adult central nervous system. Brain research reviews 2005;48:196-206.

[29] Soulet D, Rivest S. Microglia. Current Biology 2008;18:R506-R8.

[30] Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. Annual review of immunology 2009;27:119-45.

[31] Polazzi E, Monti B. Microglia and neuroprotection: from in vitro studies to therapeutic applications. Progress in neurobiology 2010;92:293-315.

[32] Kim SU, de Vellis J. Microglia in health and disease. Journal of neuroscience research 2005;81:302-13.

[33] Hanisch U-K, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature neuroscience 2007;10:1387-94.

[34] Napoli I, Neumann H. Microglial clearance function in health and disease. Neuroscience 2009;158:1030-8.

[35] David S, Kroner A. Repertoire of microglial and macrophage responses after spinal cord injury. Nature Reviews Neuroscience 2011;12:388-99.

[36] Saijo K, Glass CK. Microglial cell origin and phenotypes in health and disease. Nature Reviews Immunology 2011;11:775-87.

[37] Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity 2005;23:344-6.

[38] Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annual review of immunology 2009;27:451-83.

[39] Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. The Journal of Neuroscience 2009;29:13435-44.

[40] Durafourt BA, Lambert C, Johnson TA, Blain M, Bar-Or A, Antel JP. Differential responses of human microglia and blood-derived myeloid cells to FTY720. Journal of neuroimmunology 2011;230:10-6.

[41] Ransohoff RM, Cardona AE. The myeloid cells of the central nervous system parenchyma. Nature 2010;468:253-62.

[42] Graeber MB, Streit WJ. Microglia: biology and pathology. Acta neuropathologica 2010;119:89-105.

[43] Lindvall O, Kokaia Z, Bengzon J, Elme E, Kokaia M. Neurotrophins and brain insults. Trends in neurosciences 1994;17:490-6.

[44] Nakajima K, Kohsaka S. Microglia: activation and their significance in the central nervous system. Journal of biochemistry 2001;130:169-75.

[45] Schwartz M, Butovsky O, Brück W, Hanisch U-K. Microglial phenotype: is the commitment reversible? Trends in neurosciences 2006;29:68-74.

[46] Kitamura Y, Yanagisawa D, Takata K, Taniguchi T. Neuroprotective function in brain microglia. Current Anaesthesia & Critical Care 2009;20:142-7.

[47] Streit WJ, Conde JR, Fendrick SE, Flanary BE, Mariani CL. Role of microglia in the central nervous system's immune response. Neurological research 2005;27:685-91.

[48] Suzumura A, Sawada M, Yamamoto H, Marunouchi T. Transforming growth factor-beta suppresses activation and proliferation of microglia in vitro. The Journal of Immunology 1993;151:2150-8.

[49] Minghetti L, Levi G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Progress in neurobiology 1998;54:99-125.

[50] Blaylock R. Chronic Microglial Activation andExcitotoxicity Secondary to Excessive Immune Stimulation: Possible Factors in Gulf War Syndrome and Autism. Journal of American Physicians and Surgeons 2004;9.

[51] Banati RB, Gehrmann J, Schubert P, Kreutzberg GW. Cytotoxicity of microglia. Glia 1993;7:111-8.

[52] Lee SC, Dickson DW, Liu W, Brosnan CF. Induction of nitric oxide synthase activity in human astrocytes by interleukin-1β and interferon-γ. Journal of neuroimmunology 1993;46:19-24.

[53] Chao CC, Hu S. Tumor necrosis factor-alpha potentiates glutamate neurotoxicity in human fetal brain cell cultures. Developmental neuroscience 1994;16:172-9.

[54] Meda L, Cassatella MA, Szendrei GI, Otvos L, Baron P, Villalba M, et al. Activation of microglial cells by β-amyloid protein and interferon-γ. Nature 1995;374:647-50.

[55] Tambuyzer BR, Ponsaerts P, Nouwen EJ. Microglia: gatekeepers of central nervous system immunology. Journal of leukocyte biology 2009;85:352-70.

[56] Théry C, Chamak B, Mallat M. Cytotoxic effect of brain macrophages on developing neurons. European Journal of Neuroscience 1991;3:1155-64.

[57] Brown GC. Nitric oxide and neuronal death. Nitric oxide 2010;23:153-65.

[58] Loane DJ, Byrnes KR. Role of microglia in neurotrauma. Neurotherapeutics 2010;7:366-77.

[59] Rosenberg GA. Matrix metalloproteinases in neuroinflammation. Glia 2002;39:279-91.

[60] Rutland-Brown W, Langlois JA, Thomas KE, Xi YL. Incidence of traumatic brain injury in the United States, 2003. The Journal of head trauma rehabilitation 2006;21:544-8.

[61] Bhatia M, Ramnath RD, Chevali L, Guglielmotti A. Treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against acute pancreatitis. American Journal of Physiology-Gastrointestinal and Liver Physiology 2005;288:G1259-G65.

[62] Liu T, Zhang T, Yu H, Shen H, Xia W. Adjudin protects against cerebral ischemia reperfusion injury by inhibition of neuroinflammation and blood-brain barrier disruption. J Neuroinflammation 2014;11:107.

[63] Ajmone-Cat MA, Bernardo A, Greco A, Minghetti L. Non-steroidal anti-inflammatory drugs and brain inflammation: effects on microglial functions. Pharmaceuticals 2010;3:1949-65.

[64] Fischer CD, Beatty JK, Duquette SC, Morck DW, Lucas MJ, Buret AG. Direct and indirect anti-inflammatory effects of tulathromycin in bovine macrophages: inhibition of CXCL-8 secretion, induction of apoptosis, and promotion of efferocytosis. Antimicrobial agents and chemotherapy 2013;57:1385-93.

[65] Yamada J, Jinno S. Alterations in neuronal survival and glial reactions after axotomy by ceftriaxone and minocycline in the mouse hypoglossal nucleus. Neuroscience letters 2011;504:295-300.

[66] Bi W, Zhu L, Wang C, Liang Y, Liu J, Shi Q, et al. Rifampicin inhibits microglial inflammation and improves neuron survival against inflammation. Brain research 2011;1395:12-20.

[67] Slatore CG, Tilles SA. Sulfonamide hypersensitivity. Immunology and allergy clinics of North America 2004;24:477-90.

[68] Ciriaco M, Ventrice P, Russo G, Scicchitano M, Mazzitello G, Scicchitano F, et al. Corticosteroid-related central nervous system side effects. Journal of pharmacology & pharmacotherapeutics 2013;4:S94.

[69] Beg S, Swain S, Hasan H, Barkat MA, Hussain MS. Systematic review of herbals as potential anti-inflammatory agents: Recent advances, current clinical status and future perspectives. Pharmacognosy reviews 2011;5:120.

[70] Grill MF, Maganti RK. Neurotoxic effects associated with antibiotic use: management considerations. British journal of clinical pharmacology 2011;72:381-93.

[71] Heppner FL, Roth K, Nitsch R, Hailer NP. Vitamin E induces ramification and downregulation of adhesion molecules in cultured microglial cells. Glia 1998;22:180-8.

[72] Bellezzo JM, Leingang KA, Bulla GA, Britton RS, Bacon BR, Fox ES. Modulation of lipopolysaccharide-mediated activation in rat Kupffer cells by antioxidants. Journal of Laboratory and Clinical Medicine 1998;131:36-44.

[73] Numakawa Y, Numakawa T, Matsumoto T, Yagasaki Y, Kumamaru E, Kunugi H, et al. Vitamin E protected cultured cortical neurons from oxidative stress-induced cell death through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Journal of neurochemistry 2006;97:1191-202.

[74] Sinha J, Das N, Basu M. Liposomal antioxidants in combating ischemia-reperfusion injury in rat brain. Biomedicine & pharmacotherapy 2001;55:264-71.

[75] Devaraj S, Li D, Jialal I. The effects of alpha tocopherol supplementation on monocyte function. Decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. Journal of Clinical Investigation 1996;98:756.

[76] Shirley R, Ord EN, Work LM. Oxidative stress and the use of antioxidants in stroke. Antioxidants 2014;3:472-501.

[77] Love S. Oxidative stress in brain ischemia. Brain Pathology 1999;9:119-31.

[78] SUGIYAMA Y, FUJITA T, MATSUMOTO M, OKAMOTO K, IMADA I. Effects of idebenone (CV-2619) and its metabolites on respiratory activity and lipid peroxidation in brain mitochondria from rats and dogs. Journal of pharmacobio-dynamics 1985;8:1006-17.

[79] Nagy IZ-. Chemistry, toxicology, pharmacology and pharmacokinetics of idebenone: a review. Archives of gerontology and geriatrics 1990;11:177-86.

[80] Yamazaki N, Kiyota Y, Take Y, Miyamoto M, Nagawa Y, Nagaoka A. Effects of idebenone on memory impairment induced in ischemic and embolization models of cerebrovascular disturbance in rats. Archives of gerontology and geriatrics 1989;8:213-24.

[81] Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, et al. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. Journal of Clinical Investigation 2002;109:661-70.

[82] Otsuka M, Tsuchiya S, Aramaki Y. Involvement of ERK, a MAP kinase, in the production of TGF-β by macrophages treated with liposomes composed of phosphatidylserine. Biochemical and biophysical research communications 2004;324:1400-5.

[83] Zhang J, Fujii S, Wu Z, Hashioka S, Tanaka Y, Shiratsuchi A, et al. Involvement of COX-1 and up-regulated prostaglandin E synthases in phosphatidylserine liposome-induced prostaglandin E 2 production by microglia. Journal of neuroimmunology 2006;172:112-20.

[84] Krieger M. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annual review of biochemistry 1994;63:601-37.

[85] Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 1997;89:1121-32.

[86] Krahling S, Callahan MK, Williamson P, Schlegel RA. Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages. Cell death and differentiation 1999;6:183-9.

[87] Ravichandran KS. “Recruitment signals” from apoptotic cells: invitation to a quiet meal. Cell 2003;113:817-20.

[88] Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. Cold Spring Harbor perspectives in biology 2013;5:a008748.

[89] Verhoven B, Krahling S, Schlegel RA, Williamson P. Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. Cell death and differentiation 1999;6:262-70.

[90] Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RAB, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 2000;405:85-90.

[91] Huynh M-LN, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. The Journal of clinical investigation 2002;109:41-50.

[92] De Simone R, Ajmone-Cat MA, Nicolini A, Minghetti L. Expression of Phosphatidylserine Receptor and Down-Regulation of Pro-Inflammatory Molecule Production by its Natural Ligand in Rat Microglial Cultures. Journal of Neuropathology & Experimental Neurology 2002;61:237-44.

[93] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nature reviews Drug discovery 2005;4:145-60.

[94] Storm G, Crommelin DJ. Liposomes: quo vadis? Pharmaceutical Science & Technology Today 1998;1:19-31.

[95] Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacological reviews 1999;51:691-744.

[96] Van Rooijen N, Sanders A. Kupffer cell depletion by liposome-delivered drugs: Comparative activity of intracellular clodronate, propamidine, and ethylenediaminetetraacetic acid. Hepatology 1996;23:1239-43.

[97] Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. The Journal of clinical investigation 2012;122:787-95.

[98] Fukasawa M, Adachi H, Hirota K, Tsujimoto M, Arai H, Inoue K. SRB1, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells. Experimental cell research 1996;222:246-50.

[99] Schroit AJ, Fidler IJ. Effects of liposome structure and lipid composition on the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. Cancer research 1982;42:161-7.

[100] Allen T, Austin G, Chonn A, Lin L, Lee K. Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochimica et Biophysica Acta (BBA)-Biomembranes 1991;1061:56-64.

[101] Aramaki Y, Akiyama K, Hara T, Tsuchiya S. Recognition of charged liposomes by rat peritoneal and splenic macrophages: effects of fibronectin on the uptake of charged liposomes. European journal of pharmaceutical sciences 1995;3:63-70.

[102] Geelen T, Yeo SY, Paulis L, Starmans L, Nicolay K, Strijkers GJ. Internalization of paramagnetic phosphatidylserine-containing liposomes by macrophages. J Nanobiotechnol 2012;10:1-.

[103] Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nature Reviews Immunology 2013;13:621-34.

[104] Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, et al. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like

receptor 4-dependent pathway. Proceedings of the National Academy of Sciences 2003;100:8514-9.

[105] Aramaki Y, Nitta F, Matsuno R, Morimura Y, Tsuchiya S. Inhibitory effects of negatively charged liposomes on nitric oxide production from macrophages stimulated by LPS. Biochemical and biophysical research communications 1996;220:1-6.

[106] Kamal-Eldin A, Appelqvist L-Å. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 1996;31:671-701.

[107] Djordjević VB. Free radicals in cell biology. International review of cytology 2004;237:57-89.

[108] Qin L, Liu Y, Wang T, Wei S-J, Block ML, Wilson B, et al. NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. Journal of Biological Chemistry 2004;279:1415-21.

[109] Scott AJ, O'Dea KP, O'Callaghan D, Williams L, Dokpesi JO, Tatton L, et al. Reactive oxygen species and p38 mitogen-activated protein kinase mediate tumor necrosis factor α-converting enzyme (TACE/ADAM-17) activation in primary human monocytes. Journal of Biological Chemistry 2011;286:35466-76.

[110] Hashioka S, Han Y-H, Fujii S, Kato T, Monji A, Utsumi H, et al. Phosphatidylserine and phosphatidylcholine-containing liposomes inhibit amyloid β and interferon-γ-induced microglial activation. Free Radical Biology and Medicine 2007;42:945-54.

[111] Ajmone-Cat M, De Simone R, Nicolini A, Minghetti L. Effects of phosphatidylserine on p38 mitogen activated protein kinase, cyclic AMP responding element binding protein and nuclear factor-κB activation in resting and activated microglial cells. Journal of neurochemistry 2003;84:413-6.

[112] Takasugi Y, Kurai F, Kazume I, Otsuka M, Negishi Y, Tada R, et al. Down Regulation of MyD88 in Macrophages Treated with Liposomes Composed of Phosphatidylserine. 2013.

[113] Devaraj S, Jialal I. α-Tocopherol decreases tumor necrosis factor-α mRNA and protein from activated human monocytes by inhibition of 5-lipoxygenase. Free Radical Biology and Medicine 2005;38:1212-20.

[114] Haller D, Holt L, Kim SC, Schwabe RF, Sartor RB, Jobin C. Transforming growth factor-β1 inhibits non-pathogenic gramnegative bacteria-induced NF-κB recruitment to the interleukin-6 gene promoter in intestinal epithelial cells through modulation of histone acetylation. Journal of Biological Chemistry 2003;278:23851-60.

[115] Yang H, Paromov V, Smith M, Stone WL. Preparation, characterization, and use of antioxidant-liposomes. Advanced Protocols in Oxidative Stress I: Springer; 2008. p. 277-92.

CHAPTER 3

Suppression of atopic dermatitis in mice model by reducing inflammation using PS-coated biodegradable microparticles

3.1 Introduction

3.1.1 Atopic dermatitis

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease characterized by itching and redness on the dorsal area of the skin. The prevalence of this disease has increased rapidly among the children over the recent decades [1]. It has a significant impact on the quality of life of patients and their families. AD is characterized by impaired skin barrier function caused by genetic reasons and environmental factors such as tissue damage, allergen exposure, and dysbiosis of skin microbiome [2]. The activation of T lymphocytes, dendritic cells, macrophages, keratinocytes, mast cells and eosinophils are characteristic of the AD skin inflammatory responses. Among these cells, macrophages show various functions both in acute and chronic phase of AD inflammation [3].

3.1.2 Macrophage in atopic dermatitis

In AD, macrophages are known to accumulate in acutely and chronically inflamed skin. During the early and short inflammatory phase, macrophages exert pro-inflammatory functions like antigen-presenting phagocytosis and the production of inflammatory cytokines and growth factors that facilitate the resolution of inflammation. However, persistence of pro-inflammatory activity and altered phenotype of macrophages result in the

development of chronic inflammatory phase of AD. In the AD lesion, since both the pro-inflammatory and anti-inflammatory cytokines are secreted, both M1 and M2 are expected to exist [3].

3.1.2 Drugs targeting atopic dermatitis

Topical agents are the mainstay of AD therapy which include corticosteroids, calcineurin inhibitors, antimicrobials/antiseptics, antihistamines and others (eg, coal tar, phosphodiesterase inhibitors) [4].

3.1.2.1 Anti-inflammatory drugs for atopic dermatitis

Among the anti-inflammatory drugs topical corticosteroids are most frequently used in the management of AD in both adults and children and are the mainstay of anti-inflammatory therapy. But chronic use of these drugs have some adverse side effects such as cutaneous purpura, telangiectasia, striae, focal hypertrichosis, acneiform or rosacea-like eruptions, and skin atrophy [5, 6], hyperglycemia and hypertension [7].

3.1.2.2 Phosphatidylserine and atopic dermatitis

Liposomes containing PS found to function as a mimicry of the apoptotic cells which leads macrophage to secret anti-inflammatory cytokines [8]. Thus, PS-containing liposomes have been used for the suppression of the inflammation such as skin edema [9] inflammatory bone lose [10] and myocardial infarction [11]. For the AD treatment, Sur et al reported the pioneering work. They examined oral administration of the PS-containing aqueous dispersion to the mice with the atopy-like dermatitis which was prepared by a synthetic

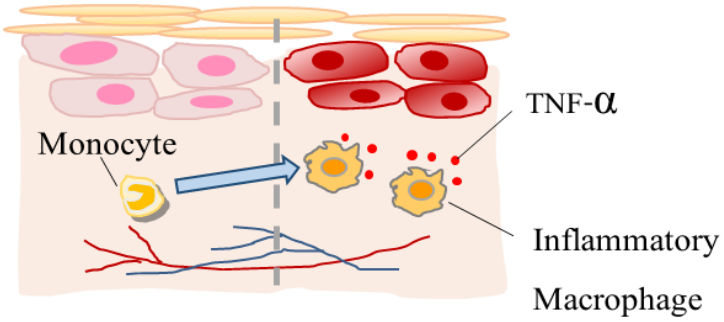
hapten (2, 4-dinitroflurobenzen) [12]. They found reduction of the inflammatory cytokine production and suppression of the skin inflammation.

3.1.3 Polymeric lipid microparticle to improve atopic dermatitis therapy

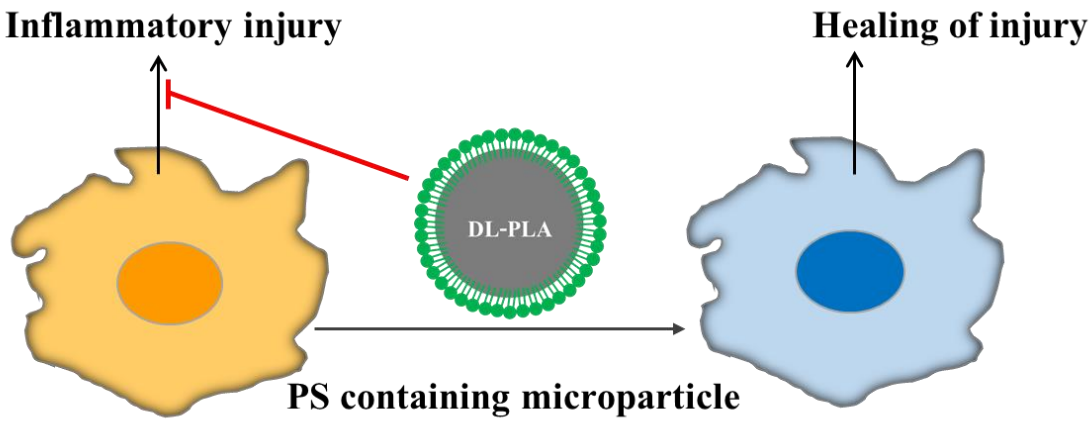
Polymeric lipid microparticle composed of Poly Lactic acid (PLA) as the core and PS arranged outside could be a potential drug carrier at the site of inflammation to induce an anti-inflammatory response by targeting the macrophages. Because of the phagocytic activity of macrophages, this PS-coated particle can be expected to be taken up by the macrophage and used as an efficient vehicles for the intracellular delivery of drugs. The hydrophobic nature of the particle could be an efficient carrier of hydrophobic drugs.

3.1.4 Overview of this chapter

In this chapter we focused on the "anti-inflammatory response" of the cell by the secretion of the anti-inflammatory cytokines. For this purpose I prepared a microparticle composed of Poly-D, L-Lactic acid (PLA) as the core and PS arranged outside. The microparticle containing PS should change the inflammatory phenotype of the macrophage into anti-inflammatory by binding with PS through the bridging molecule MFG-E8 and integrin.



By this way, the inflammation caused by the release of inflammatory cytokines from the macrophage will be reduced by the secretion of anti-inflammatory cytokines. This can be explained more precisely by the diagram show below.



The microparticle coated with PS is a new formulation with large hydrophobic core possibly accommodating hydrophobic drug inside and expected longer retention in the body. The particle is thought to cure inflammation by activating the different signaling pathways. The large size of the particle can also elongate the anti-inflammatory response in the body. The mechanical stability of the particle is achieved due to the filled polymer core (Fig.1).

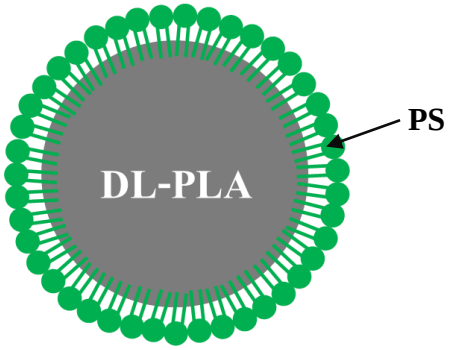


Fig. 1. Structure of phospholipid-coated microparticle with biodegradable PLA core.

3.2 Results and discussions

3.2.1 Microparticle preparation

The lipid-coated microparticles with PLA core were obtained by emulsification. D, L-mixture of LPA was used for the particle preparation to accelerate the hydrolytic decomposition in tissue environment. The average size of the micro particles were determined by the particle counter analyzer to be 1.4 and 0.99 μm for PS and PC-coated ones, respectively. The ζ-potential values of the microparticle were found to be -40.6±0.6 and -5.2±0.5 mV for PS and PC-coated ones, respectively. The negative ζ-potential of the PS-coated one indicated successful coating with the negatively charged PS on the surface.

3.2.2 Cellular uptake of the microparticle

Apoptotic cells and PS-containing liposomes are known to be engulfed by macrophage *via* PS receptors such as MFG-E8/integrin $\alpha_v\beta_3$ and Tim4 [13].

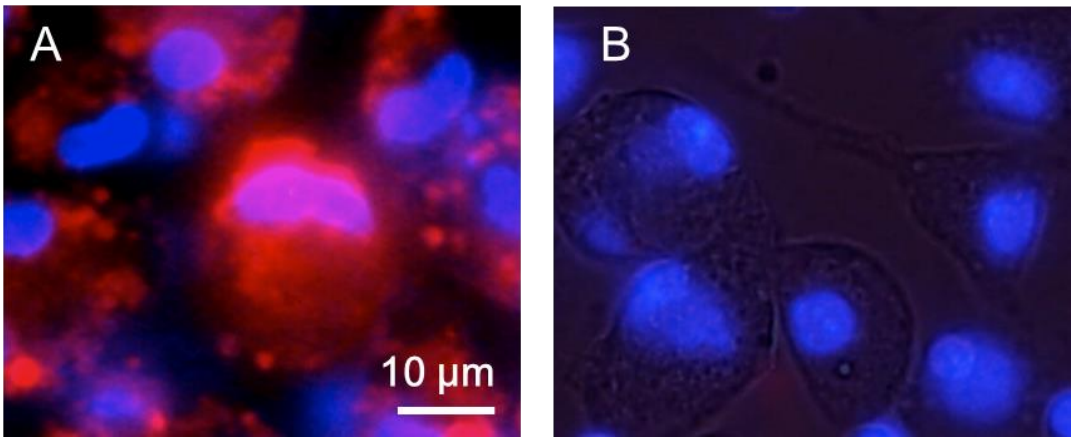


Fig. 2. Uptake of PS-coated (A) and PC-coated microparticle (B) by mouse peritoneal macrophage observed by fluorescent microscopy. Microparticles were labelled with PKH26 shown as red. Blue color is nucleus stained by Hoechst.

Here we examined whether the PS-coated microparticle can be engulfed by mouse peritoneal macrophages by fluorescence microscopy after encapsulating PKH26. As shown in Figure 2, the PS-coated microparticle was significantly taken up by the macrophages, while the uptake of the PC-coated microparticle was negligible. These results indicate the microparticle surface is covered with PS with recognizable orientation on the surface by the PS receptors.

3.2.3 Effect of PS-coated microparticle on TNF-α production

The alteration of the peritoneal macrophages to anti-inflammatory phenotype in response to the recognition of PS presented on the microparticle was speculated from the change of TNF-α secretion from the peritoneal macrophages. The TNF-α production is known to be one of the typical marker of inflammatory M1 macrophage.

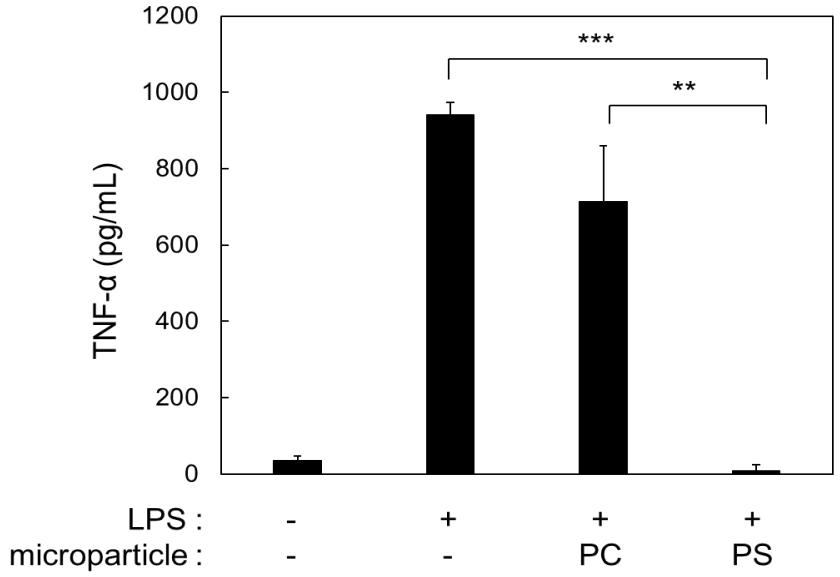


Fig. 3. Effects of PS-coated microparticle on secretion of TNF-α from peritoneal macrophage. Peritoneal macrophage was stimulated with LPS in the presence or absence of each microparticle. ***$p < 0.001$, **$p<0.01$.

As shown in Figure 3, the LPS-stimulation activates the macrophage to produce TNF-α. However, the significant decrease of the TNF-α production was observed in the presence of the PS-coated microparticles. Such significant change was not observed in the macrophages pretreated with the PC-coated one. Taken together, these results indicate that the treatment with the PS-coated microparticles suppressed the alteration of the macrophage phenotype to the anti-inflammatory one.

3.2.4 Treatment of AD model mice with PS-microparticle

We prepared AD model mice by applying the commercial ointment, which includes extracts of house dust mite, to the dorsal skin behind the neck every second day. After the third application (day 0 of Figure 4), the dermatitis score of the mice became 3 on average. Then the application of the microparticle dispersion was started *via* subcutaneous injection. Figure 4 shows macroscopic features of representative mice in day 6 and 12 of each treatment. In the case of the mice with non-treated and with the PC-coated microparticles, the skin lesion continued for 12 days. On the other hand, the mice treated with the PS-coated microparticles showed clear improvement of the lesion and excoriation disappeared on day 12. The time dependent change of the average score of 4 mice for each group was summarized in Figure 5. The dermatitis score was not change significantly after treatment of the PC-coated microparticle. However, in the case of the PS-coated microparticle, the score gradually decreased with the progress of the treatment and reacted to below 1 after 10 days. This confirms the therapeutic effect of the PS-coated microparticle for AD.

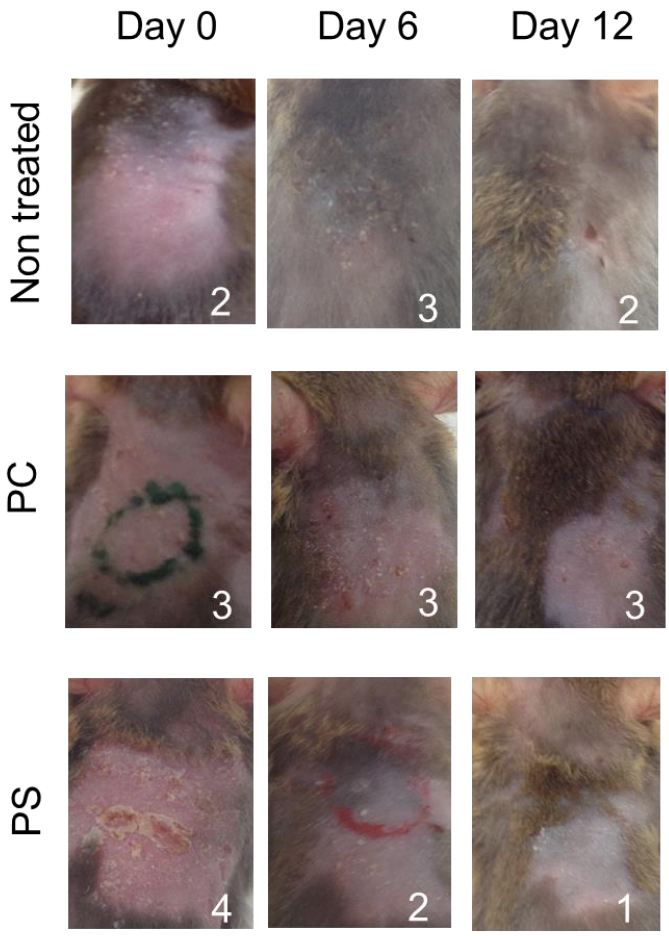


Fig. 4. Macroscopic features of AD lesion of the representative NC/Nga mouse treated with microparticles on every second day for 7 times. Day zero is the first day of the microparticle treatment. Suspension of microparticle (100 μL) was subcutaneously injected in the dermatitis area.

As mentioned in the introduction, AD lesions contain both M1 and M2 macrophages, but the exact role of M1 and M2 macrophage in acute and chronic inflammation of AD is not clear [3]. However, it is a fact that macrophage numbers increase in acute and chronic AD skin lesions [14].

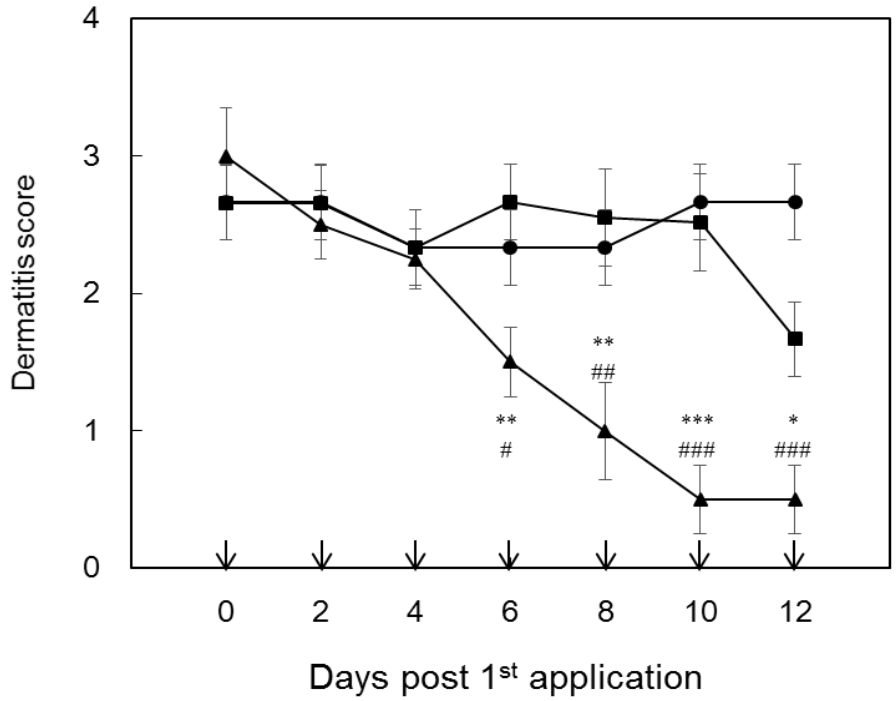


Fig 5. Dermatitis score of NC/Nga mice with AD during treatment of each microparticle. A suspension (100 μL) of PS-coated (closed triangles) and PC-coated microparticle (closed squares) were subcutaneously injected in the dermatitis area. The concentration of the suspension is 27 mg/mL of microparticle, containing 125 μg/mL of PC or PS. A group of the mice was not treated with any medication (closed circles). Each data represents the mean ± S.E. of 4 mice. The significant scores, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ are shown where PS-coated microparticle treated group compared with PC treated group and $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ when compared with non-treated group.

Several studies have reported increased levels of pro-inflammatory cytokines including TNF-α in serum, lymph nodes, and skin lesions of NC/Nga mice treated with house dust mites such as Df or *Dermatophagoides pteronyssinus* (Dp) [15-18]. Among pro-inflammatory cytokines, the TNF-α plays a pivotal role in the occurrence of AD, for example, through the induction of keratinocyte-derived thymic stromal lymphopoietin production that can trigger dendritic cell-mediated Th2 inflammatory responses [19-21].

Furthermore, there is a significant correlation between serum TNF-α and histamine levels in both AD animal models [16] and patients [22]. In this context, reduction of pro-inflammatory cytokines including TNF-α is an efficient way to treat AD [19, 23]. In the present study, PS-coated microparticles decreased the TNF-α production in LPS-stimulated peritoneal macrophages. Therefore, inhibitory effect of PS-coated microparticles on the TNF-α production in macrophages may lead to the reduction of AD symptoms in NC/Nga mice treated with Df. Although further studies are needed to clarify these mechanisms, our study suggests that PS-coated microparticles have the therapeutic effect on the AD.

3.3. Conclusion

Here we developed a treatment of the AD model mice by using the PS-coated microparticles. The microparticle can be recognized by the macrophage leading to the reduction of the inflammatory cytokine production. The microparticle coated with PS is a new formulation with large hydrophobic core possibly accommodating hydrophobic drug inside and expected longer retention in the injected area. Potential drugs could be incorporated into PLA core of the particle such as glucocorticoids, non-steroidal anti-inflammatory drugs, and antibiotics.

3.4 Experiment

3.4.1 Chemicals

3-sn-Phosphatidyl-L-serine sodium from bovine and L-α Phosphatidylcholine from egg yolk were purchased from Sigma Aldrich (Japan). Poly (D, L-lactide) ester terminated (Mw 10,000-18,000) was also purchased from Sigma Aldrich (Japan). Polyvinyl alcohol (Mw 89,000-98,000) was purchased from Sigma Aldrich (Japan). TNF-α Cytokine Quantification ELISA Kits were purchased from RnD systems (Japan). Biostir AD was purchased from Biostir.

3.4.2 Experimental Animals

Female C5BL/6 mouse, 6 week old were purchased from Kyudo Company Ltd and female NC/Nga mice were purchased from Charles River Japan (Japan). These mice were all housed under conditions of controlled temperature (20-26^{o}C) and humidity (30-70%) and lighting. Food and tap water were provided twice a week. All mice were reared up to 8-10 weeks of age. The animal experiments were performed in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan).

3.4.3 Microparticle Preparation

A chloroform solution (200 µL) containing 25 mg/mL poly (D, L-lactide) was placed in 15 mL tube. Then on the chloroform solution, an aqueous solution (2.5 mL) containing polyvinyl alcohol (0.4 mg/mL), phosphatidylserine (1.16 mg/mL, 1.4 mM), and HEPES (10 mM, pH 7.4) was placed. Then the two-phase solution was vigorously mixed for

emulsification for 1.5 h in vacuum to evaporate chloroform. The resulting PS-coated microparticle was collected by centrifugation at 300 g and washed three times with HEPES buffer (10 mM, pH 7.4). Finally the PS-coated microparticle was obtained as dispersion in HEPES buffer and stored at 5 °C. PC-coated microparticle was prepared similarly by using PC instead of PS in aqueous phase. Fluorescence-labeled microparticle was prepared similarly by mixing PKH26 (7 μM) in chloroform phase.

3.4.4 Microparticle characterization

Microparticle size was confirmed by Particle Counter Analyzer (CDA-1000). The ζ-potential of the microparticle is measured at 25ºC with a Zeta Size Analyzer. The results were reported as the average of five runs.

3.4.5 Peritoneal macrophage

Peritoneal macrophages was collected from the peritoneum cavity of the mouse (C5BL/6N, female, 6 week-old) injected with 3% thioglycolate containing PBS 7-days before the extraction. The collected macrophage was cultured in a RPMI-1640 medium (Wako) containing10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco Invitrogen Co.), in a humidified atmosphere containing 5% CO_2 at 37 °C.

3.4.6 Cellular uptake of microparticle

Freshly collected peritoneal macrophages were seeded on the 96 well plate with $5X10^3$ cells/well. After culturing for 24 hours, the cells were gently washed with the medium to

remove the floating cells to obtain a single layer of the macrophage. The PS- or PC-coated microparticles labelled with PKH26 were added to the cells as 2.6 mg/mL (containing 125 µg/mL of phospholipid) in culture medium containing 10% FBS. After one hour, the cellular uptake of the particles was confirmed by fluorescence microscopy.

3.4.7 Measurement of TNF-α production

For evaluating the secretion of the inflammatory cytokine we used peritoneal macrophage. The macrophages were extracted from C5BL/6N mice using the above mentioned protocol and were centrifuged at 300G for 5 min and suspended in fresh medium of DMEM+FBS. Macrophages were seeded on the 24 well plate with $2X10^4$ cells/well. Then, the medium containing microparticles 2.6 mg/mL (containing 125 µg /mL of phospholipid) was added and cells were incubated for 24 hours at 37^{o}C. Then, lipopolysaccharides (LPS) dissolved in DMEM without FBS (10 µg/mL) were added and further incubated for of 3 hours. The cell culture supernatant were collected for cytokine detection after 3 hours and then TNF-α were quantified by ELISA (RnD systems Japan) according to the manufacturer's instructions.

3.4.8 Dermatitis induction and treatment

After mice were anesthetized, their hair on the upper back was shaved with a shaver and remaining hair is removed by using depilatory cream. 4%-sodium dodecyl sulfate solution (150 µL) was applied on the shaved dorsal skin to disrupt dermal barrier [24]. Then, 100 mg of Biostir AD including Df was applied to the mice every second days for three times. If there was any hair growth on the shaved area of the mouse, it would be shaved before every treatment. Consequently, dermatitis was clearly observed. Then, 100-µL of PS-coated or

PC-coated microparticles (27 mg/mL microparticle, containing 125 μg/mL of PC or PS) were injected subcutaneously in the dermatitis area on every second day for 7 times (Fig.6).

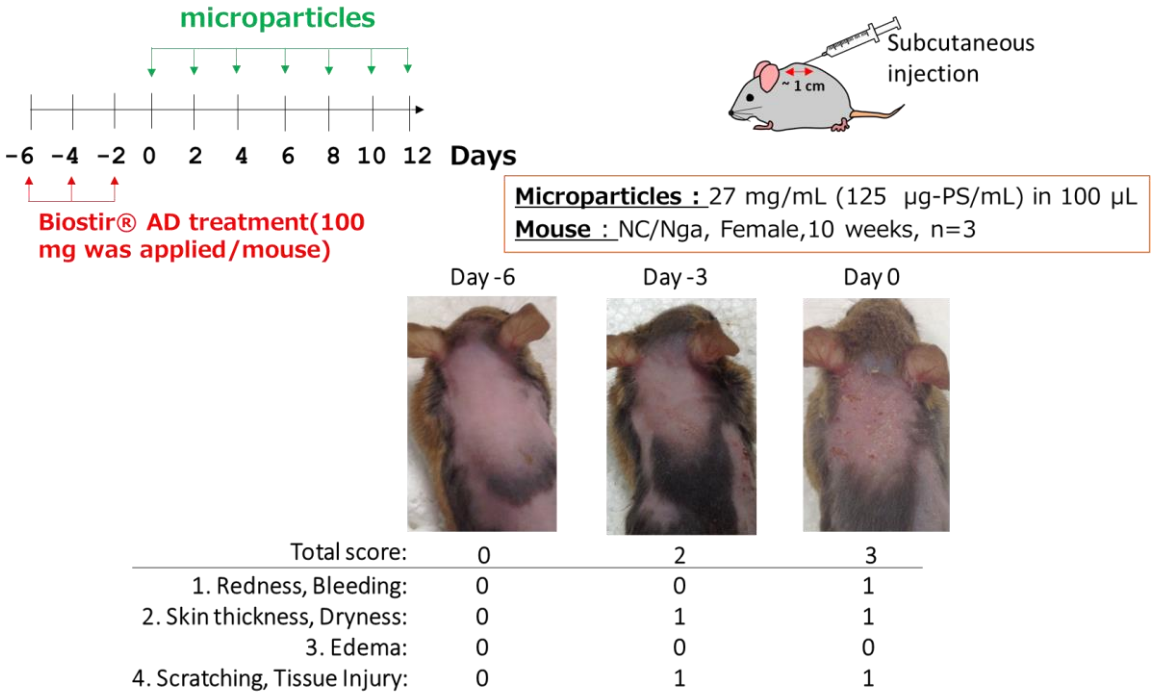


Fig.6. PS-coated microparticle treatment in AD mouse model

3.4.9 Evaluation of skin lesion

The development of dermatitis was evaluated before every elicitation by two independent researchers following the previous report [25]. The development of 1) erythema/hemorrhage 2) scarring/dryness 3) edema and 4) excoriation/erosion was scored 0 (none), 1 (mild), 2 (moderate), 3 (severe). The sum of the individual scores was taken as the dermatitis score.

3.5 Reference

[1] Moon J-S, Jeon B-S, Yoon B-I, Choi S-H, Lim C-J. Topical application of a phospholipid mixture purified from pig lungs ameliorates 2, 4-dinitrofluorobenzene-induced allergic contact dermatitis in BALB/c mice. Molecular biology reports 2012; 39: 4237-47.

[2] Bieber T. Mechanisms of Disease. N Engl J Med 2008; 358: 1483-94.

[3] Kasraie S, Werfel T. Role of macrophages in the pathogenesis of atopic dermatitis. Mediators of inflammation 2013; 2013.

[4] Eichenfield LF, Tom WL, Berger TG, Krol A, Paller AS, Schwarzenberger K, et al. Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies. Journal of the American Academy of Dermatology 2014; 71: 116-32.

[5] Callen J, Chamlin S, Eichenfield L, Ellis C, Girardi M, Goldfarb M, et al. A systematic review of the safety of topical therapies for atopic dermatitis. British Journal of Dermatology 2007;156 : 203-21.

[6] Pariser D. Topical corticosteroids and topical calcineurin inhibitors in the treatment of atopic dermatitis: focus on percutaneous absorption. American journal of therapeutics 2009;16 : 264-73.

[7] Hengge UR, Ruzicka T, Schwartz RA, Cork MJ. Adverse effects of topical glucocorticosteroids. Journal of the American Academy of Dermatology 2006; 54:1-15.

[8] Korns D, Frasch SC, Fernandez-Boyanapalli R, Henson PM, Bratton DL. Modulation of macrophage efferocytosis in inflammation. Frontiers in immunology 2011; 2.

[9] Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends in immunology 2004; 25: 677-86.

[10] Ma HM, Wu Z, Nakanishi H. Phosphatidylserine-containing liposomes suppress inflammatory bone loss by ameliorating the cytokine imbalance provoked by infiltrated macrophages. Laboratory investigation 2011; 91: 921-31.

[11] Harel-Adar T, Mordechai TB, Amsalem Y, Feinberg MS, Leor J, Cohen S. Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. Proceedings of the National Academy of Sciences 2011; 108:1827-32.

[12] Sur B-J, Lee B, Yeom M, Han J-J, Choi H-D, Lee H, et al. Inhibitory effect of phosphatidylserine on atopy-like dermatitis in NC/Nga mice. Food Science and Biotechnology 2010; 19:1513-8.

[13] Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. The Journal of clinical investigation 2012; 122: 787-95.

[14] Kiekens R, Thepen T, Oosting A, Bihari I, Van de Winkel J, Bruijnzeel-Koomen C, et al. Heterogeneity within tissue-specific macrophage and dendritic cell populations during cutaneous inflammation in atopic dermatitis. British Journal of Dermatology 2001; 145: 957-65.

[15] Kang JS, Lee K, Han S-B, Ahn J-M, Lee H, Han MH, et al. Induction of atopic eczema/dermatitis syndrome-like skin lesions by repeated topical application of a crude extract of Dermatophagoides pteronyssinus in NC/Nga mice. International immunopharmacology 2006; 6:1616-22.

[16] Sung Y-Y, Yoon T, Jang JY, Park S-J, Jeong G-H, Kim HK. Inhibitory effects of Cinnamomum cassia extract on atopic dermatitis-like skin lesions induced by mite antigen in NC/Nga mice. Journal of Ethnopharmacology 2011; 133: 621-8.

[17] Sung Y-Y, Lee AY, Kim HK. The Gardenia jasminoides extract and its constituent, geniposide, elicit anti-allergic effects on atopic dermatitis by inhibiting histamine in vitro and in vivo. Journal of ethnopharmacology 2014; 156: 33-40.

[18] Yang G, Choi C-H, Lee K, Lee M, Ham I, Choi H-Y. Effects of Catalpa ovata stem bark on atopic dermatitis-like skin lesions in NC/Nga mice. Journal of ethnopharmacology 2013;145: 416-23.

[19] Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. Journal of Clinical Investigation 2004; 113: 651.

[20] Carmi-Levy I, Homey B, Soumelis V. A modular view of cytokine networks in atopic dermatitis. Clinical reviews in allergy & immunology 2011; 41: 245-53.

[21] Danso MO, van Drongelen V, Mulder A, van Esch J, Scott H, van Smeden J, et al. TNF-α and Th2 Cytokines Induce Atopic Dermatitis–Like Features on Epidermal Differentiation Proteins and Stratum Corneum Lipids in Human Skin Equivalents. Journal of Investigative Dermatology 2014; 134 : 1941-50.

[22] Sumimoto S, Kawai M, Kasajima Y, Hamamoto T. Increased plasma tumour necrosis factor-alpha concentration in atopic dermatitis. Archives of disease in childhood 1992; 67: 277-9.

[23] Harskamp CT, Armstrong AW. Immunology of atopic dermatitis: novel insights into mechanisms and immunomodulatory therapies. Semin Cutan Med Surg 2013; 32: 132-9.

[24] Yamamoto M, Haruna T, Ueda C, Asano Y, Takahashi H, Iduhara M, et al. Contribution of itch-associated scratch behavior to the development of skin lesions in Dermatophagoides

farinae-induced dermatitis model in NC/Nga mice. Archives of dermatological research 2009; 301: 739-46.

[25] Yamamoto M, Haruna T, Yasui K, Takahashi H, Iduhara M, Takaki S, et al. A novel atopic dermatitis model induced by topical application with dermatophagoides farinae extract in NC/Nga mice. Allergology International 2007; 56: 139-48.

CHAPTER 4

A synergic modulation of inflammatory state of macrophages utilizing anti-oxidant and phosphatidylserine containing polymer-lipid hybrid nanoparticle (PLNP) targeting the ulcerative colitis therapy

4.1 Introduction

Ulcerative colitis (UC) is a chronic and non-specific inflammatory disorders of the gastrointestinal tract [1] involving the mucosa and sub mucosa of the colon characterized by contiguous inflammation of the colonic lamina propria with subsequent injury and disruption of the mucosal barrier. About 15% of ulcerative colitis patients experience a severe clinical course, and 30% of these patients were required colectomy [2, 3]. Furthermore, prolonged inflammation of the intestinal tract reduces patients' quality of life and increases the possibility of colon cancer development [4].

4.1.1 Inflammatory process in ulcerative colitis

In ulcerative colitis inflammation is limited primarily to the mucosa and consists of continuous involvement of variable severity with ulceration, edema, and hemorrhage along the length of the colon. The characteristic findings are acute and chronic inflammation of the mucosa by polymorphonuclear leukocytes and mononuclear cells. Multiple factors such as over production of pro-inflammatory mediators including reactive oxygen species (ROS) [5, 6], nitric oxide [7], cytokines (e.g. IL-6, TNF-α) [8], arachidonate metabolites, produced by infiltration of activated macrophages in the lamina propria have been involved in the

pathogenesis of ulcerative colitis [7, 9, 10]. Such factors exceeds the intestinal defense system, leading to exacerbating of intestinal injury in ulcerative colitis [11] (Fig.1).

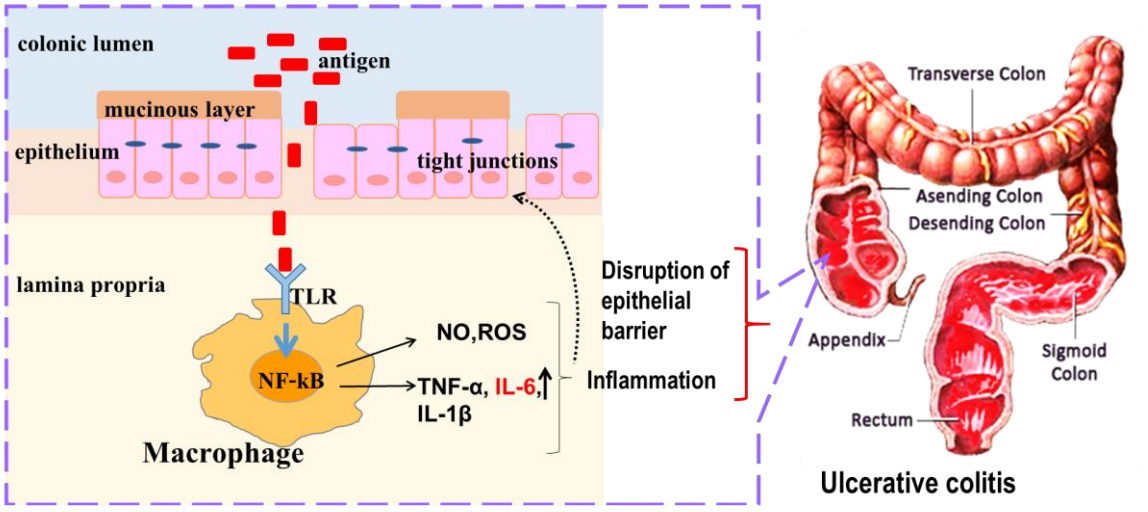


Fig.1. Pathophysiology of ulcerative colitis

4.1.1.1 Macrophage in intestinal inflammation

Monocytes and macrophages play pivotal roles in the host innate and adaptive immunological responses, inflammatory responses, and tissue repair and remodeling. They mediate their functions by interacting directly with microorganisms or other host cells or via secretion of a wide range of products [12] such as cytokines that exert their potent biological effects after binding to specific cell surface receptors. The production and release of pro-inflammatory mediators by macrophage play the prime role in the physiopathology of UC. There is strong evidence that the inflammatory microenvironment modulates intestinal wound healing [13, 14]. Macrophages, which exhibit a great plasticity, are important components of the inflamed tissue and constitute an important element of regenerative responses [15, 16] in intestine.

4.1.1.2 Macrophage polarization

Macrophages reveal a great plasticity and have been substantially divided into two subsets based on receptor expression, effector function and cytokine production [17, 18]. M1, or classically activated, macrophages are characterized by the expression of high levels of pro-inflammatory cytokines such as TNF-α, IL-6, IL-β1, and mediate antitumor immunity and defense of the host from microorganisms. On the other hand, M2, or alternatively activated, macrophages express high levels of anti-inflammatory cytokines and dampen inflammation, promote tissue remodeling and repair, wound healing, help in parasite clearance and tumor progression, and possess immune regulatory functions as described in previous chapter.

4.1.2 Oxidative stress and ulcerative colitis

In the body, cells are continuously threatened by the damage with reactive oxygen/nitrogen species (ROS/RNS), which are produced during physiological oxygen metabolism. Both ROS and RNS at low or moderate concentrations play as signaling molecules and involve in defense response against infectious agents. However, excessive production of ROS and RNS or their inefficient scavenging leads to oxidative stress. This condition is potentially dangerous as it may alter inflammatory response and lead to lipid and protein modifications, DNA damage, apoptosis, cancerogenic cell transformation [19-21] and implicate a number of human diseases, including inflammatory bowel diseases such as ulcerative colitis and colorectal cancer.

4.1.2.1 Generation of free radicals and reactive oxygen and nitrogen species

The reactive oxygen species (ROS) are the most common factors for describing oxidative stress, although it is not only the contributor of this stress. Nitrogen species, such as nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$) also play the important role in the cascade of signaling and oxidative injury [22, 23].

The most plentiful free radical in human tissues is the superoxide anion ($O_2^{\bullet -}$), produced by one electron reduction to molecular oxygen [24] via mitochondrial electron transport chain which converts about 1–3% of total oxygen to the superoxide anion [25]. Another source of this superoxide anion is an enzymatic reaction catalyzed by xanthine oxidase (XO) and membrane enzyme complexe NADPH oxidases. In the gastrointestinal tract, superoxide anion is mainly generated by xanthine oxidase.

Hydrogen peroxide (H_2O_2) functions as a radical intermediate but it is not a free radical itself. It is an important compound because of its ability to penetrate and diffuse across cellular membranes [26]. It can be reduced to hypochlorous acid through myeloperoxidases [27], or into hydroxyl radicals via Fenton reaction [28]. The hydroxyl radical ($^{\cdot}OH$) is another damaging radical to biological systems [29, 30] which is produced from multiple sources (Table 1).

Another important group of free radicals are reactive nitrogen species (RNS) that are byproducts of nitric oxide synthases (NOS). In the intestinal sub-mucosa and mucosal regions the NOS metabolizes arginine to citrulline and forms the nitric oxide radical ($NO^{\bullet}$) via a five-electron oxidative reaction [31]. The inducible NOS (iNOS) produces $NO^{\bullet}$ in a

constant manner in inflamed tissue and is responsible for an excessive generation of RNS in activated macrophages, leukocytes and epithelial cells in the intestinal mucosa [32].

Table 1. Outlines the common free radicals, their sources, and the accompanying cellular defense systems [34-38].

Reactive molecule	Source	Cellular defense mechanisms
Superoxide ($O2^{\cdot-}$)	Electron transport chain Cyclooxygenase NADPH-oxidase Xanthine Oxidase	Superoxide Dismutase
Hydrogen peroxide (H_2O_2)	Glucose oxidase NADPH-oxidase Superoxide dismutase P450 reductase Xanthine oxidase	Catalase Glutathione peroxidase Myeloperoxidase
Hydroxyl ($^{\cdot}OH$)	Fenton chemistry H_20_2 degradation	Glutathione
Nitric Oxide (NO)	Nitric oxide synthase	Glutathione
Peroxynitrile($ONOO^{\cdot}$)	Reaction with nitric oxide	

In UC, the activation of iNOS, cyclooxygenase-2, 5-lipooxygenase increased the production of NO, prostaglandin E2 and they contribute to a damage of intestinal mucous membrane by the overproduction of free radicals and impairment of anti-oxidative system [33]. The reaction between $NO^{\bullet}$ with $O_2^{\bullet -}$ leads to peroxynitrite production ($ONOO^{-}$), which is an aggressive oxidizing agent that causes DNA fragmentation, lipid oxidation, dysfunction and cell death of intestinal mucosa.

4.1.2.2 Endogenous antioxidant defense mechanism in intestine

A low or non-harmful concentration of both ROS and RNS are sustained by the anti-oxidative defense mechanisms in the intestine. The enzymes involved in anti-oxidative mechanism include catalase, superoxide dismutase and glutathione peroxidase. The other non-enzymatic endogenous and exogenous scavengers like glutathione (GSH), transient ions such as Fe^{2+}, Cu^{2+} or flavonoids have role in anti-oxidative mechanism.

4.1.3 Drugs targeting for ulcerative colitis

The treatment goal of ulcerative colitis is to maintenance of remission and most of the treatment modalities comprise anti-inflammatory therapy.

4.1.3.1 Anti-inflammatory drugs

The drugs primarily used for the treatment of ulcerative colitis include, 5-aminosalicylic acid, sulfasalazine, steroids, and immunosuppressive drugs such as azathioprine, 6-mercaptopurine and monoclonal antibody [39-41]. These drugs exert their anti-inflammatory roles by inhibiting both COX activity and NF-kB/ MAPK p38 signaling in activated macrophages [42, 43]. However, the use of these drugs have some side effects [44, 45] and 20% to 40% of ulcerative colitis patients do not respond to these conventional therapy and may receive secondary drug treatment or colectomy [46]. As a result, various therapeutic strategies have been studied for curing of ulcerative colitis targeting the inflammatory pathways.

4.1.3.2 Antioxidants

Recently, some antioxidants have been shown to exert anti-inflammatory activity in ulcerative colitis model. The antioxidants induce their anti-inflammatory role by scavenging of free radicals to neutralize ROS and NOS as well as inhibiting transcription of NF-kB from the activated macrophages [47, 48]. The α-tocopherol and *N*-acetylcysteine have been reported to attenuate chemically induced colitis [49, 50] by inhibiting the activation of macrophages [48].

4.1.3.3 Phosphatidylserine mediated anti-inflammation

Phosphatidylserine (PS) is another promising molecule that has anti-inflammatory effects in activated macrophages [51] by enhancing the clearance of apoptotic cells. After the phagocytosis, macrophages actively raise the anti-inflammatory response by decreasing the production of pro-inflammatory cytokines [52].

4.1.4 Polymer-lipid hybrid nanoparticles to improve the drug delivery

In recent years, the use of various nanoparticles have been increased in every branch of medicine for their ability to deliver drugs in the optimum dosage range, to increase therapeutic efficacy of the drug, or to alleviate the side effects. Polymers (polymeric nanoparticles, polymeric micelles, dendrimers) [53-55], lipids (liposomes, lipid nanoparticles) [56-58], and metals (gold, silica) [59, 60] have been commonly used as nanocarriers. Among the nanocarriers, the most prominent are the polymeric nanoparticles and liposomes, attributed to their advantageous characteristics [61, 62].

Compared with polymeric nanoparticles, the liposomes have been thought as more ideal drug delivery vehicles due to their superior biocompatibility as liposomes are basically analogues of biological membranes, and it can be prepared from both natural and synthetic phospholipids [63]. But liposomes are easily cleared by the reticuloendothelial system leading to their poor bioavailability [64]. On the other hand polymeric nanoparticles enhance the bioavailability due to their formulation [65, 66].To consider the limitations of polymeric nanoparticles and liposomes, recently a new generation delivery vehicle of therapeutics named lipid–polymer hybrid nanoparticles has been developed [67] which combine the characteristics of both polymeric nanoparticles and liposomes.

4.1.5 Polymer-lipid nanoparticle therapy targeting macrophage activity

The nanoparticle formulations that are easily taken up by macrophages would be highly advantageous for macrophage-targeting drug delivery [68]. Because of the phagocytic activity of macrophages, polymer-lipid hybrid nanoparticle can be used as efficient vehicles for the intracellular delivery of drugs. After the uptake by macrophage, the phospholipid bilayers are disrupted by phospholipase and the release of the encapsulated drug lead to an intracellular accumulation of the drug [69] in the macrophages.

4.1.6 Overview of this chapter

Macrophage is one of the most abundant leucocytes in the intestinal mucosa where they are essential for maintaining homeostasis. However, it is also implicated in the pathogenesis of disorders such as ulcerative colitis, a chronic inflammatory bowel disease. The normal colon mucosa plays an immune, endocrine, and barrier function. Injuries occurring in the intestinal mucosa insult its barrier function, increased intestinal mucosal permeability allows microbes and antigens to invade and excessively stimulate immune response, triggering intestinal inflammation. Excessive ROS/RNS are produced leading to oxidative stress during the inflammatory response, exaggerating inflammatory lesions in the pathogenesis of UC. In this chapter, I focused on decreasing of oxidative stress and inflammatory response produced by activated macrophages using a combination of anti-oxidants and anti-inflammatory molecule.To achieve this target, we designed a simple biodegradable polymer-lipid hybrid nanoparticle (PLNP) (Fig.2) which comprising PLA as polymer core and phospholipid-PVA as the shell, and exhibited a complementary characteristics of both polymeric nanoparticles and liposomes, particularly in terms of their physical stability and biocompatibility.

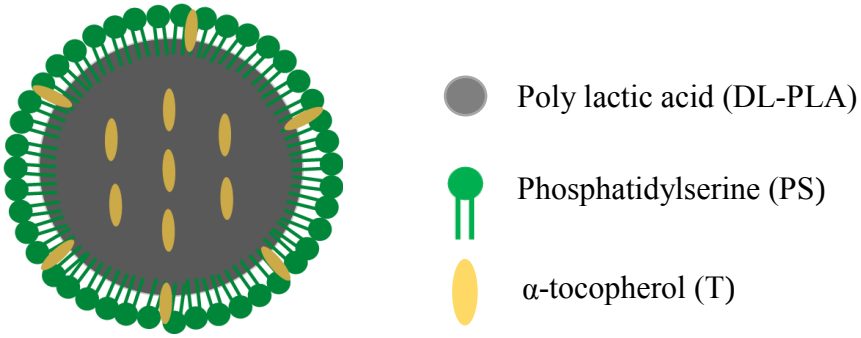
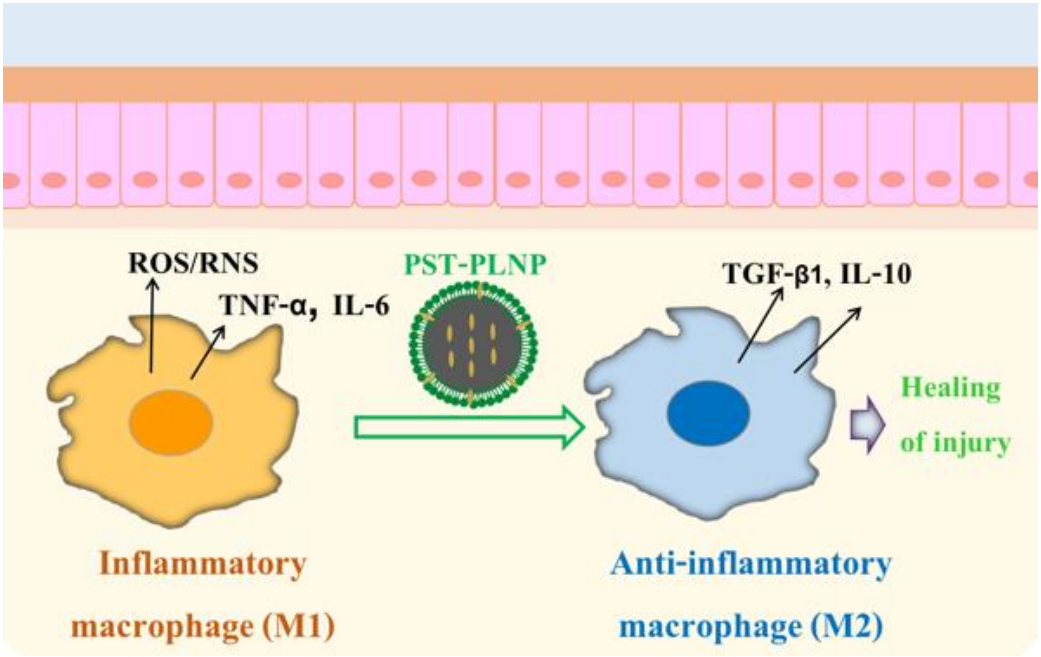


Fig.2. α-tocopherol and PS containing polymer-lipid hybrid nanoparticle (PST-PLNP)

CHAPTER 4

To suppress the oxidative stress and inflammation in ulcerative colitis, this is the first time we proposed α-tocopherol and phosphatidylserine combination in the polymer-lipid hybrid nanoparticle (PST-PLNP) and investigated their consequent up-regulation of anti-inflammatory response in activated macrophages.



4.2 Result and Discussion

4.2.1 Characterization of PLNPs

We prepared four kinds of PLNPs by emulsification/evaporation technique. The characteristic values of the PLNPs are summarized in Table 2. The average diameter of the PC-, PCT-, PS- and PST-PLNPs were 411, 445,410 and 390 nm and their ζ-potentials were -1.2, -6.9, -13.3 and -15.4 mV, respectively. The size and shape of the prepared PLNPs were confirmed by SEM images (Fig. 4).The negative ζ-potentials of the PS- and PST-PLNP reflect the negatively charged PS in the PLNPs. Representative dynamic light scattering spectra for the four PLNPs are shown in Fig. 3. All the PLNPs have similar sizes and relatively narrowly distributed diameters.

Table 2. Size, ζ-potential and PdI values of the PLNPs in 10 mM HEPES buffer solution at pH 7.4.

Name of PLNP	Organic phase				Water phase	Particle size (nm)	ζ-potential (mV)	PdI
	PS (1%)	PC (1%)	DL-PLA (2.5%)	α-tocopherol (2%)	PVA (2%)			
PC	-	1 mg	10 mg	-	100 mg	411 ± 6.75	-1.18 ± 0.11	0.31 ± 0.03
PCT	-	1 mg	10 mg	0.6 mg	106 mg	445 ± 9.38	- 6.9 ± 0.23	0.21 ± 0.02
PS	1 mg	-	10 mg	-	100 mg	410 ± 4.48	-13.3 ± 1.06	0.24 ± 0.01
PST	1 mg	-	10 mg	0.6 mg	106 mg	390 ± 1.03	-15.4 ± 1.84	0.16 ± 0.01

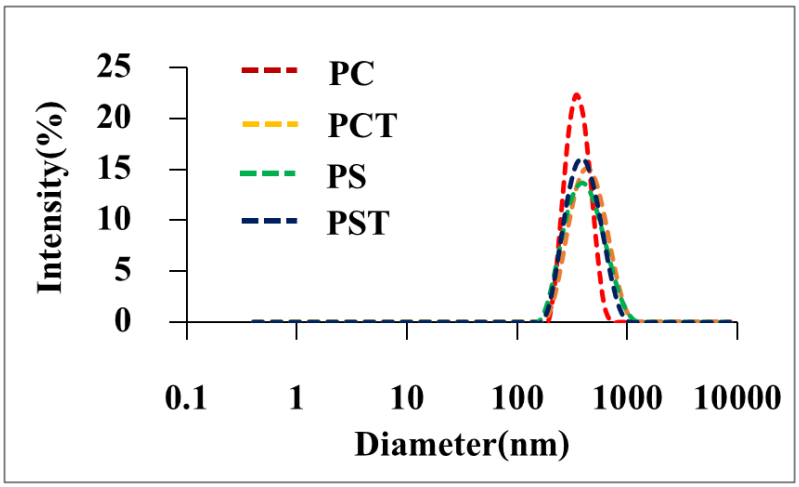


Fig. 3. Size distributions of the prepared PLNPs.

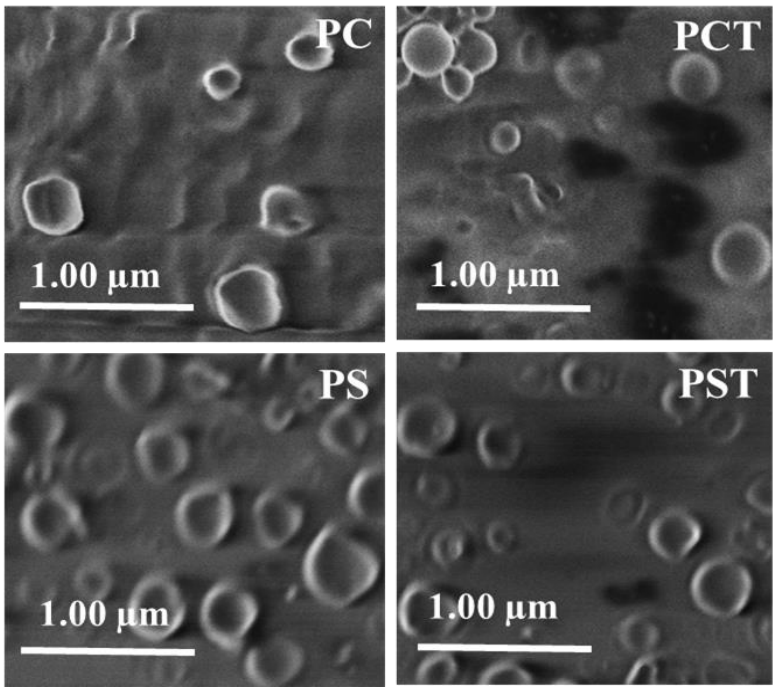


Fig.4. Scanning electron microscopic **(**SEM) images of the prepared PLNPs

4.2.2 PLNPs uptake by RAW 264.7 macrophages

Uptake of the PLNPs by LPS-treated RAW 264.7 macrophages was investigated with fluorescence microscopy (Fig. 5) after the fluorescence labelling (DiD) of the particles. Red fluorescence was observed in cytosol of the macrophages after incubation with PCT-, PS- and PST-PLNPs, indicating that these fluorescently labelled PLNPs had been engulfed by the cells. The PCT-PLNP was engulfed by the macrophages due to the formation of quinone by α-tocopherol [70].

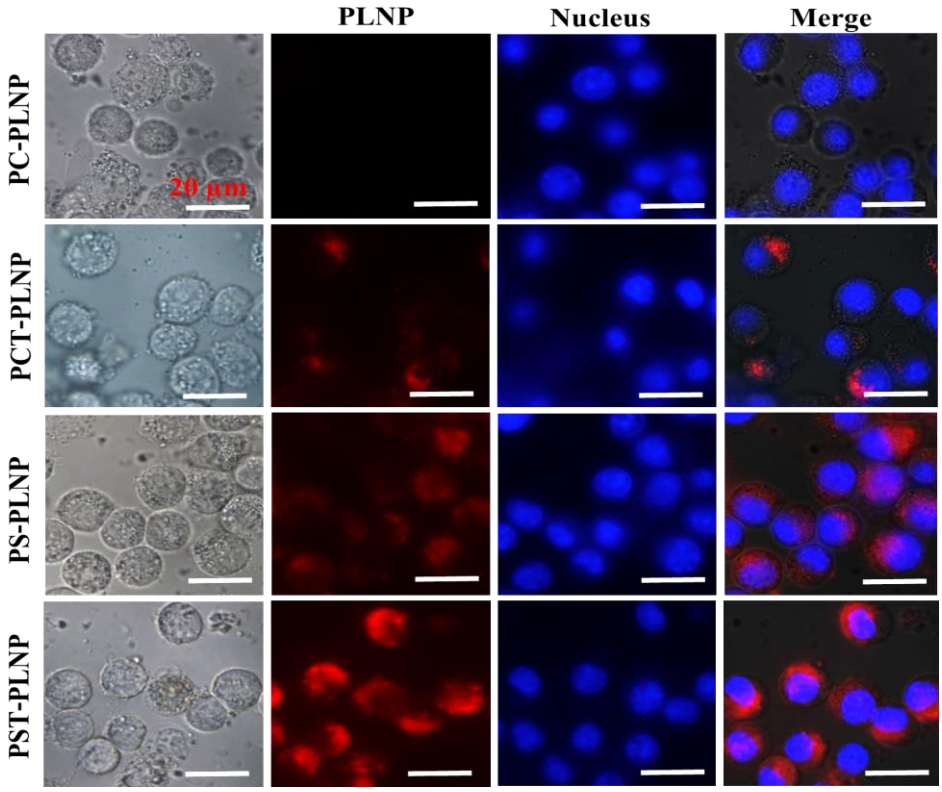


Fig. 5. Uptake of PLNPs by RAW 264.7 macrophages. Macrophages were treated with LPS (1 µg/ml) and PLNPs (125 µg lipid/mL) and incubated for 3 h at 37 °C. The images were taken with a fluorescence microscope (blue color is the nucleus, after Hoechst staining, and red indicates PLNPs, labelled with DiD).

As the cellular uptake of PC-PLNP was almost negligible, we concluded that the significant uptake of both PS- and PST-PLNPs are resulted from PS-specific mediated endocytosis [71-76].

4.2.3 Cell survival assay of macrophages incubated with PLNPs

We examined effects of PLNPs addition on the ability of macrophages to resist lipopolysaccharide (LPS) after the 24 and 48h incubation. As shown in Fig.6, PLNPs did not have any remarkable effect on macrophages survival for 24 h incubation as compared with control. However, the viability of macrophages significantly differed with different PLNPs treatment for 48 h incubation.

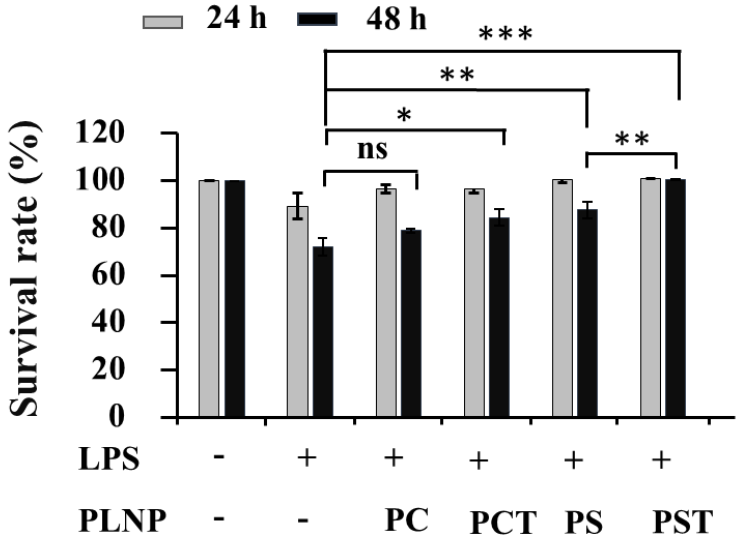


Fig. 6. RAW 264.7 macrophages viability in LPS stimulation. Macrophages (1×10^4 cells/well) were seeded in 96 well plates, treated with PLNPs (125 µg lipid/ml) and LPS (1 µg/ml) and incubated for 24 h and 48 h. Absorbance were measured at 450 nm with the microplate reader at the indicated time. Data are means ± S.D. ns, not significant; *, $p < 0.05$**, $p < 0.01$; ***, $p < 0.001$.

Viability of the macrophages improved in the presence of α-tocopherol and PS in PCT, PS and PST-PLNPs. The PST-PLNP showed the most significant effect in increasing the cell survival. The superiority of PST-PLNP resulted from a synergistic effects of α-tocopherol and PS, both conferring resistance against LPS stimulation of the macrophages.

4.2.4 Suppression of NO production by activated macrophages when treated with anti-inflammatory and anti-oxidative PLNP

Activated macrophages produce NO, which reacts with ROS to propagate the injury in colitis area [7]. Excess production of NO exacerbates the clinico-pathological features of UC by direct cytotoxicity, activation of leukocytes [77], vasodilatation, reduced smooth muscle tone [78], increased production of nitrosamines [79] and interaction with superoxide to form the highly toxic peroxynitrite radical [80].

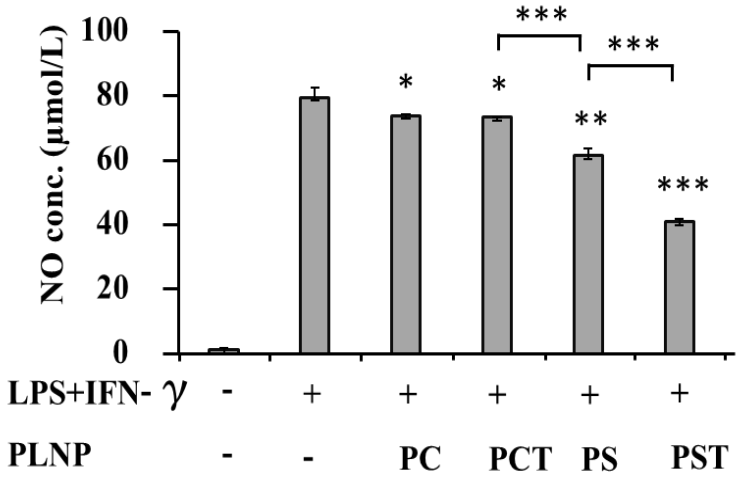


Fig. 7. Effects of PLNPs (125 µg lipid/ml) on NO production by RAW 264.7 macrophages stimulated with LPS (1 µg/ml) + IFN-γ (100 U/ml). Data are means ± S.D. ns, not significant; *, p< 0.05; **, p< 0.01; ***, *p* < 0.001.

Thus, suppression of NO production is important to prevent the oxidative stress and colitis injury in colon. Here we examined inhibitory effects of PLNPs on NO production from activated macrophages. To enhance NO production, the macrophages were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/mL) [81]. As shown in Fig. 7, PC- and PCT-PLNPs had less effect on NO production. In contrast, significant suppression of NO production was observed with both PS- and PST-PLNPs treatments, while the greatest effect showed by the PST-PLNP. Previously, NO suppressing effects of PS [82] in activated peritoneal macrophages was reported. Our findings confirmed that combination of PS and α-tocopherol in PST-PLNP have synergistic effect, compared with those having only PS or α-tocopherol, at reducing NO production from activated macrophages.

4.2.5 Reduction of IL-6 production by treatment with anti-inflammatory PLNP

To examine the anti-inflammatory activity of each PLNP preparation, we measured production of IL-6, a typical pro-inflammatory cytokine that is known to be secreted from macrophages in ulcerative colitis [83]. We had found that pro-inflammatory signalling produced by LPS stimulation increased the production of IL-6 from macrophages through trans-signaling by STAT3 molecule (signal transducer and activator of transcription-3) [84]. As shown in Fig.8, PC-PLNP had no effect on IL-6 production from LPS activated macrophages. In contrast, significant inhibition of IL-6 production was found in PCT-, PS- and PST-PLNP treatments. Among all the treatments, the greatest effect on IL-6 production was observed by PST-PLNP treatment. α-tocopherol is also known to decrease IL-6 production [85, 86].

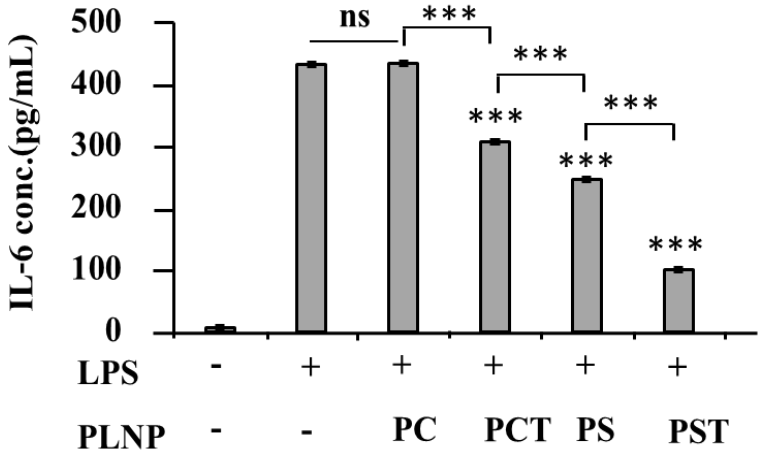


Fig. 8. Effects of PLNPs (125 μg lipid/ml) on IL-6 production by macrophages stimulated with LPS (1 μg/ml). Data are means ± SD of three separate experiments. ns, not significant; ***, $p < 0.001$.

Our results indicated a clear synergy between PS and α-tocopherol due to their dominant effect on reduction of the IL-6 production compared to PCT- and PS-PLNP.

4.2.6 Effects of anti-inflammatory PLNP on TGF-β1 production by activated macrophages

We next assessed effects of PST-PLNP on production of the anti-inflammatory cytokine TGF-β1, a key regulator in the maintenance of immune and inflammatory responses. Evidence suggests that TGF-β acts together with growth factors in protecting host tissue from luminal changes and can help the mucosal healing in IBD [87, 88]. Inflammation is inhibited by TGF-β1, which acts as a negative regulator of NF-kB activation [89]. As shown in Fig.9, both PS- and PST-PLNP significantly increased TGF-β1 production from activated macrophage, whereas PC-and PCT-PLNPs were not effective.

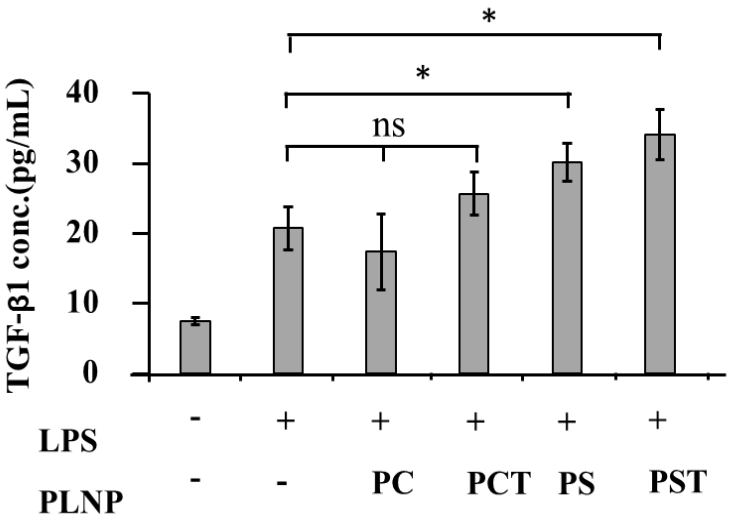


Fig. 9. Effects of PLNPs (125 μg lipid/ml) on TGF-β1 production by macrophages stimulated by LPS (1 μg/mL). Data are means ± SD of three separate experiments. ns, not significant; *, $p<0.05$.

Whether α-tocopherol has an additional effect on the production of TGF-β1 in activated macrophage was not clear in this experiment. This result is consistent to our previous result [90].

4.3 Conclusion

In this chapter, I reported the preparation of potential anti-oxidative and inflammatory property bearing simple biodegradable polymer-lipid hybrid nanoparticle (PLNP) comprising PLA as polymer core and phospholipid-PVA as shell which exhibits a complementary characteristics of both polymeric nanoparticles and liposomes, particularly in terms of their physical stability and biocompatibility. To attain the expected result, we incorporated α-tocopherol and phosphatidylserine in the particle and investigated their consequent up-regulation of anti-inflammatory response in activated macrophages. These two components showed clear synergistic anti-inflammatory and anti-oxidative effects on activated macrophages. PST-PLNP significantly increased viability of activated macrophages. Our data also demonstrated that PST-PLNP are able to alter the inflammatory properties of macrophages by inhibiting NO and inflammatory cytokine production when those cells are stimulated. The particle is also able to enhance the production of anti-inflammatory cytokine in activated macrophages. The potential anti-inflammatory and anti-oxidative effects of this particle indicates the inflammatory modulation of the macrophages. Therefore, this α-tocopherol and phosphatidylserine containing polymer-lipid hybrid nanoparticle could be a potential drug carrier and effective approach for threating the chronic inflammatory diseases such as ulcerative colitis targeting the macrophage activity.

4.4 Experiment

4.4.1 Materials

3-sn-Phosphatidyl-L-serine sodium from bovine brain and L- α- Phosphatidylcholine from egg yolk and lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111; B4) were purchased from Sigma Aldrich (Japan). Poly (D, L-lactide) ester terminated (Mw18, 000-28,000) and polyvinyl alcohol (Mw 89,000-98,000) were also purchased from Sigma Aldrich (Japan). α-tocopherol was purchased from TCI. ELISA-kits for mouse IL-6 and TGF-β1 were purchased from RnD systems. Nitric oxide assay kit (Griess reagent) was purchased from Dojindo Co. Japan.

4.4.2 Preparation of polymer lipid hybrid nanoparticle (PLNP)

A dichloromethane-toluene mixed (1.1 g/cm^3) solution containing 400 µL PLA (D, L-lactide) (25 mg/mL), 100 µL phosphatidylserine (10 mg/mL) and 30 µL α-tocopherol (20 mg/mL) were placed in 1.5 mL tube and mixed by vortex. Then this organic solution was added onto the aqueous solution (5.3 mL) containing 2% polyvinyl alcohol (20 mg/mL). Then the two-phase solution was vigorously mixed by vortex for 1 min and sonication for 5 min at 40 output. Then the organic solvent was evaporated from the solution by reduced pressure using oil vacuum pump for 2.5 h with shaking. The resulting polymeric lipid nanoparticle was collected by centrifugation at 10,000 rpm at 4^0C for 10 min. Then the particle was washed three times with distilled water. Fluorescence-labeled lipid nanoparticle was prepared similarly by mixing with DiD (6 µg/mL) in organic phase.

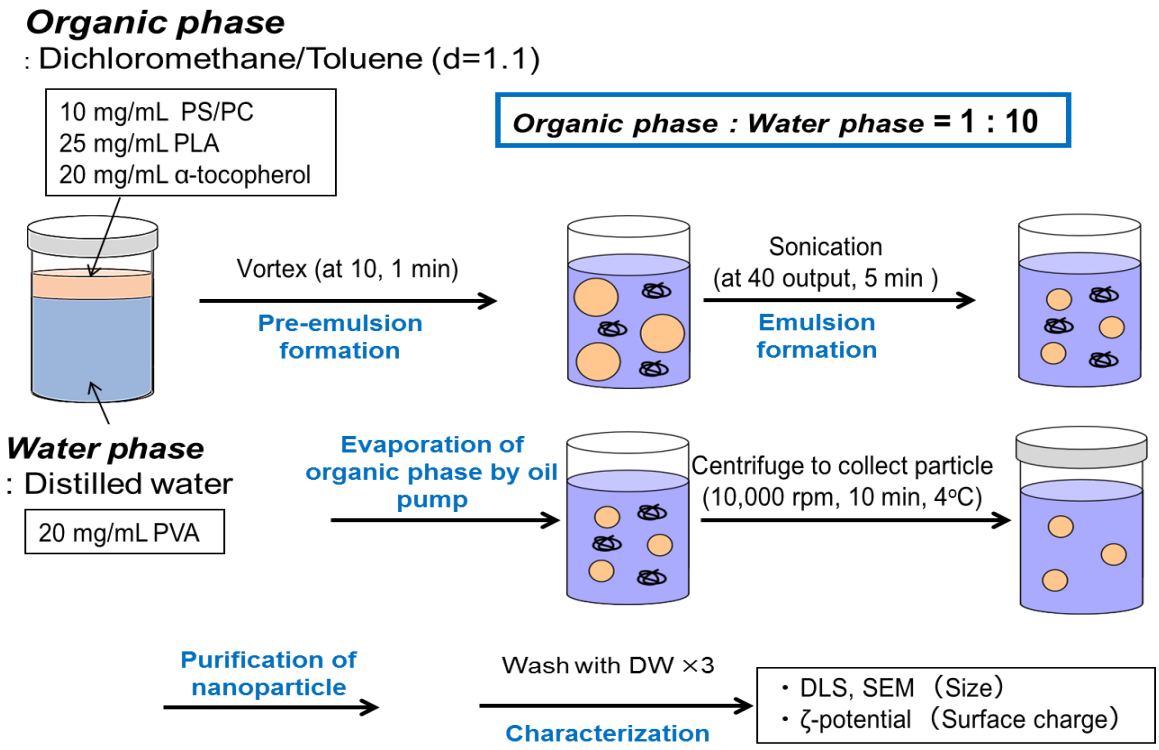


Fig.10. Preparation of PLNP by emulsification/evaporation technique

4.4.3 Characterization of the nanoparticles

3.4.3.1 Determination of particle size, polydispersity index, and zeta potential

The size, polydispersity index, and ζ-potential of the PLNPs are measured with a dynamic light-scattering spectrophotometer (Zetasizer Nanoseries, Malvern Instruments, UK) at 25 °C. For measuring the size, polydispersity index, and ζ-potential, the samples were suspended in 10 mM HEPES buffer at pH 7.4 by vortex and the obtained results were reported as the average of three runs.

4.4.3.2 Scanning electron microscopy (SEM) of nanoparticles

The morphology of the nanoparticles was investigated by SEM (SU8000, HITACHI, Japan). Briefly, after collection, the particles were washed three times with distilled water. After complete removing of the water, the particles were kept in desiccator for 48 h. Then the dried sample were placed on SEM plate coated with carbon for taking the SEM images.

4.4.4 Cell culture

RAW 264.7 murine macrophage (DS Pharma Biomedical, Japan) was maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/ mL amphotericin B (all from Gibco Invitrogen Co., NY, USA).The cells was harvested in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

4.4.5 Inflammatory activation of macrophages

For the assay of activation of macrophage with and without PLNPs, we treated cells with lipopolysaccharide (1 µg/mL) and interferon-γ (100 U/mL) [81].

4.4.6 Cellular uptake of PLNPs

Internalization of DiD labeled PLNPs in macrophages was observed by fluorescent microscopy (BZ-8100, Keyence, Japan). Briefly, RAW 264.7 macrophages were seeded in the petri dish with DMEM high containing FBS. After the cell became 70-80 % confluent then they were added to the 96 well glass surface plate. 1×10^4 cells were added in 100 µl of DMEM containing FBS per well. After 24 h of incubation the cells were washed two times

with DPBS. The particles and LPS were added in 100 µL of DMEM without FBS. Next, after 3 h of incubation at 37^0C the cells were observed for the uptake of the particles by fluorescence microscopy.

4.4.7 Assay for cell survival

Relative cell viability was measured using cell counting kit-8 (WST-8, Dojindo Laboratories, Inc. Japan). Briefly, 1×10^4 RAW 264.7 cells were seeded in 100 µL of DMEM containing FBS in 96 well plate. After 24 h of incubation the medium were replaced with FBS free DMEM high. Then the particles and LPS were added and incubated for 24 or 48 h at 37^0C. Thereafter, 10 µL of WST-8 was added to the each well after the indicated period and incubated for 3 h at 37^0C. Conversion of WST-8 into formazan by living cells was measured using micro plate reader (Wallac ARVO.SX 1420 Multilabel counter) at 450 nm absorbance. Total number of living cells were expressed as a percentage relative to untreated control samples.

4.4.8 NO quantification

The accumulation of NO_2^-, a stable end product, extensively used as an indicator of NO production by cultured cells, was assayed by the Griess reaction. Briefly, macrophages (1×10^5 cells/mL) were taken in 24 well plate and kept for overnight at 37^0C. Then the medium was replaced with serum and antibiotic free fresh medium and added PLNP (125 µg lipid/mL). After 1 h LPS (1 µg/mL) + IFN-γ (100 IU/mL) were added and incubated for 47 h. After a total 48h of PLNPs addition, the cell supernatant were mixed with equal volume of Griess reagent and incubated at room temperature for 15 min and subsequently

the absorbance was measured at 540 nm with a microplate reader (Wallac ARVO.SX 1420 Multilabel counter). Nitrite levels were determined using calibration line prepared by known concentrations of $NaNO_2$ as a standard.

4.4.9 Cytokine detection by ELISA

The macrophages were seeded in 24 well plate (1×10^5 cells/mL) and incubated for 24 h in at 37^0C. After 24 h the medium was replaced with serum and antibiotic free DMEM High and added PLNPs (125 µg lipid/mL). After 1 h LPS (1 µg/mL) added and kept 47 h. After a total 24 and 48 h of PLNPs addition, the cell culture supernatant was collected and cytokines were quantified by ELISA. Briefly, the sample supernatants were pipetted onto monoclonal antibody pre-coated microplate for binding with specific cytokine. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokine is added to the wells to sandwich the cytokine immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of cytokine bound in the initial step. The color development is stopped by addition of stop solution and the intensity of the color is measured by micro plate reader at 450 nm absorbance.

4.5 References

[1] Geier MS, Butler RN, Howarth GS. Inflammatory bowel disease: current insights into pathogenesis and new therapeutic options; probiotics, prebiotics and synbiotics. International journal of food microbiology 2007;115:1-11.

[2] Aratari A, Papi C, Clemente V, Moretti A, Luchetti R, Koch M, et al. Colectomy rate in acute severe ulcerative colitis in the infliximab era. Digestive and Liver Disease 2008;40:821-6.

[3] Turner D, Walsh CM, Steinhart AH, Griffiths AM. Response to corticosteroids in severe ulcerative colitis: a systematic review of the literature and a meta-regression. Clinical Gastroenterology and Hepatology 2007;5:103-10.

[4] Sunkara S, Swanson G, Forsyth CB, Keshavarzian A. Chronic inflammation and malignancy in ulcerative colitis. Ulcers 2011;2011.

[5] Araki Y, Andoh A, Fujiyama Y. The free radical scavenger edaravone suppresses experimental dextran sulfate sodium-induced colitis in rats. International journal of molecular medicine 2003;12:125-9.

[6] Bitiren M, Karakilcik AZ, Zerin M, Ozardalı I, Selek S, Nazlıgül Y, et al. Protective effects of selenium and vitamin E combination on experimental colitis in blood plasma and colon of rats. Biological trace element research 2010;136:87-95.

[7] Abdelouhab K, Rafa H, Toumi R, Bouaziz S, Medjeber O, Touil-Boukoffa C. Mucosal intestinal alteration in experimental colitis correlates with nitric oxide production by peritoneal macrophages: effect of probiotics and prebiotics. Immunopharmacology and immunotoxicology 2012;34:590-7.

[8] Mahida YR. The key role of macrophages in the immunopathogenesis of inflammatory bowel disease. Inflammatory bowel diseases 2000;6:21-33.

[9] Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology 2004;126:1620-33.

[10] De Hertogh G, Aerssens J, Geboes KP, Geboes K. Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease. World journal of gastroenterology: WJG 2008;14:845.

[11] Babbs CF. Oxygen radicals in ulcerative colitis. Free Radical Biology and Medicine 1992;13:169-81.

[12] Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. Annual Review of Pharmacology and Toxicology 1995;35:655-77.

[13] Dahan S, Roda G, Pinn D, Roth–Walter F, Kamalu O, Martin AP, et al. Epithelial: lamina propria lymphocyte interactions promote epithelial cell differentiation. Gastroenterology 2008;134:192-203. e2.

[14] Allez M, Brimnes J, Dotan I, Mayer L. Expansion of CD8+ T cells with regulatory function after interaction with intestinal epithelial cells. Gastroenterology 2002;123:1516-26.

[15] Pull SL, Doherty JM, Mills JC, Gordon JI, Stappenbeck TS. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. Proceedings of the National Academy of Sciences of the United States of America 2005;102:99-104.

[16] Lin S-L, Li B, Rao S, Yeo E-J, Hudson TE, Nowlin BT, et al. Macrophage Wnt7b is critical for kidney repair and regeneration. Proceedings of the National Academy of Sciences 2010;107:4194-9.

[17] Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in immunology 2002;23:549-55.

[18] Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. The Journal of clinical investigation 2012;122:787-95.

[19] Valko M, Morris H, Mazúr M, Rapta P, Bilton RF. Oxygen free radical generating mechanisms in the colon: do the semiquinones of vitamin K play a role in the aetiology of colon cancer? Biochimica et Biophysica Acta (BBA)-General Subjects 2001;1527:161-6.

[20] Ridnour LA, Isenberg JS, Espey MG, Thomas DD, Roberts DD, Wink DA. Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. Proceedings of the National Academy of Sciences of the United States of America 2005;102:13147-52.

[21] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology 2007;39:44-84.

[22] Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends in plant science 2002;7:405-10.

[23] Niu X, Smith CW, Kubes P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. Circulation research 1994;74:1133-40.

[24] Miller DM, Buettner GR, Aust SD. Transition metals as catalysts of "autoxidation" reactions. Free Radical Biology and Medicine 1990;8:95-108.

[25] Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. Journal of Biological Chemistry 2004;279:49064-73.

[26] Neill S, Desikan R, Hancock J. Hydrogen peroxide signalling. Current opinion in plant biology 2002;5:388-95.

[27] Sampson JB, Ye Y, Rosen H, Beckman JS. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. Archives of Biochemistry and Biophysics 1998;356:207-13.

[28] Imlay JA, Chin SM, Linn S. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science 1988;240:640-2.

[29] Cadet J, Delatour T, Douki T, Gasparutto D, Pouget J-P, Ravanat J-L, et al. Hydroxyl radicals and DNA base damage. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 1999;424:9-21.

[30] Willson RL. Hydroxyl radicals and biological damage in vitro: what relevance in vivo. Oxygen free radicals and tissue damage: Excerpta Medica Amsterdam; 1979. p. 19-42.

[31] Jomova K, Vondrakova D, Lawson M, Valko M. Metals, oxidative stress and neurodegenerative disorders. Molecular and cellular biochemistry 2010;345:91-104.

[32] Dijkstra G, Moshage H, Van Dullemen HM, de Jager-Krikken A, Tiebosch A, Kleibeuker JH, et al. Expression of nitric oxide synthases and formation of nitrotyrosine and reactive oxygen species in inflammatory bowel disease. The Journal of pathology 1998;186:416-21.

[33] Ya Sklyarov A, Panasyuk N, Fomenko I. Role of nitric oxide-synthase and cyclooxygenase/lipooxygenase systems in development of experimental ulcerative colitis. Journal of Physiology and Pharmacology 2011;62:65.

[34] Liochev SI. Reactive oxygen species and the free radical theory of aging. Free Radical Biology and Medicine 2013;60:1-4.

[35] Karpińska A, Gromadzka G. [Oxidative stress and natural antioxidant mechanisms: the role in neurodegeneration. From molecular mechanisms to therapeutic strategies]. Postepy higieny i medycyny doswiadczalnej (Online) 2012;67:43-53.

[36] Dröge W. Free radicals in the physiological control of cell function. Physiological reviews 2002;82:47-95.

[37] Sikka SC. Relative impact of oxidative stress on male reproductive function. Current medicinal chemistry 2001;8:851-62.

[38] Fridovich I. Superoxide radical and superoxide dismutases. Annual review of biochemistry 1995;64:97-112.

[39] Stange EF, Travis SP. The European consensus on ulcerative colitis: new horizons? Gut 2008;57:1029-31.

[40] Choi CH, Kim Y-H, Kim YS, Ye BD, Lee KM, Lee BI, et al. Guidelines for the management of ulcerative colitis. Intestinal Research 2012;10:1-25.

[41] Kozuch PL, Hanauer SB. Treatment of inflammatory bowel disease: a review of medical therapy. World journal of gastroenterology: WJG 2008;14:354.

[42] Williams J, Hallett M. The reaction of 5-amino-salicylic acid with hypochlorite: Implications for its mode of action in inflammatory bowel disease. Biochemical pharmacology 1989;38:149-54.

[43] Goppelt-Struebe M, Wolter D, Resch K. Glucocorticoids inhibit prostaglandin synthesis not only at the level of phospholipase A2 but also at the level of cyclo-oxygenase/PGE isomerase. British journal of pharmacology 1989;98:1287.

[44] Ciriaco M, Ventrice P, Russo G, Scicchitano M, Mazzitello G, Scicchitano F, et al. Corticosteroid-related central nervous system side effects. Journal of pharmacology & pharmacotherapeutics 2013;4:S94.

[45] Stolk JN, Boerbooms AM, de Abreu RA, de Koning DG, van Beusekom HJ, Muller WH, et al. Reduced thiopurine methyltransferase activity and development of side effects of azathioprine treatment in patients with rheumatoid arthritis. Arthritis & Rheumatism 1998;41:1858-66.

[46] Park SC, Jeen YT. Current and Emerging Biologics for Ulcerative Colitis. Gut and liver 2015;9:18.

[47] Devaraj S, Li D, Jialal I. The effects of alpha tocopherol supplementation on monocyte function. Decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. Journal of Clinical Investigation 1996;98:756.

[48] Bellezzo JM, Leingang KA, Bulla GA, Britton RS, Bacon BR, Fox ES. Modulation of lipopolysaccharide-mediated activation in rat Kupffer cells by antioxidants. Journal of Laboratory and Clinical Medicine 1998;131:36-44.

[49] Ardite E, Sans M, Panés J, Romero FJ, Piqué JM, Fernández-Checa JC. Replenishment of glutathione levels improves mucosal function in experimental acute colitis. Laboratory investigation 2000;80:735-44.

[50] Yang F, Oz HS, Barve S, de Villiers WJ, McClain CJ, Varilek GW. The green tea polyphenol (−)-epigallocatechin-3-gallate blocks nuclear factor-κB activation by inhibiting IκB kinase activity in the intestinal epithelial cell line IEC-6. Molecular Pharmacology 2001;60:528-33.

[51] Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, et al. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. Journal of Clinical Investigation 2002;109:661-70.

[52] Otsuka M, Tsuchiya S, Aramaki Y. Involvement of ERK, a MAP kinase, in the production of TGF-β by macrophages treated with liposomes composed of

phosphatidylserine. Biochemical and biophysical research communications 2004;324:1400-5.

[53] Yoo HS, Park TG. Folate receptor targeted biodegradable polymeric doxorubicin micelles. Journal of Controlled Release 2004;96:273-83.

[54] Enlow EM, Luft JC, Napier ME, DeSimone JM. Potent engineered PLGA nanoparticles by virtue of exceptionally high chemotherapeutic loadings. Nano letters 2011;11:808-13.

[55] Khandare JJ, Jayant S, Singh A, Chandna P, Wang Y, Vorsa N, et al. Dendrimer versus linear conjugate: influence of polymeric architecture on the delivery and anticancer effect of paclitaxel. Bioconjugate chemistry 2006;17:1464-72.

[56] Shmeeda H, Amitay Y, Gorin J, Tzemach D, Mak L, Ogorka J, et al. Delivery of zoledronic acid encapsulated in folate-targeted liposome results in potent in vitro cytotoxic activity on tumor cells. Journal of Controlled Release 2010;146:76-83.

[57] Xu Z, Chen L, Gu W, Gao Y, Lin L, Zhang Z, et al. The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma. Biomaterials 2009;30:226-32.

[58] Rosenthal E, Poizot-Martin I, Saint-Marc T, Spano J-P, Cacoub P, Group DS. Phase IV study of liposomal daunorubicin (DaunoXome) in AIDS-related Kaposi sarcoma. American journal of clinical oncology 2002;25:57-9.

[59] Prabaharan M, Grailer JJ, Pilla S, Steeber DA, Gong S. Gold nanoparticles with a monolayer of doxorubicin-conjugated amphiphilic block copolymer for tumor-targeted drug delivery. Biomaterials 2009;30:6065-75.

[60] Fan J, Fang G, Wang X, Zeng F, Xiang Y, Wu S. Targeted anticancer prodrug with mesoporous silica nanoparticles as vehicles. Nanotechnology 2011;22:455102.

[61] Cho K, Wang X, Nie S, Shin DM. Therapeutic nanoparticles for drug delivery in cancer. Clinical cancer research 2008;14:1310-6.

[62] Puri A, Loomis K, Smith B, Lee J-H, Yavlovich A, Heldman E, et al. Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. Critical Reviews™ in Therapeutic Drug Carrier Systems 2009;26.

[63] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nature reviews Drug discovery 2005;4:145-60.

[64] Maurer N, Fenske DB, Cullis PR. Developments in liposomal drug delivery systems. Expert opinion on biological therapy 2001;1:923-47.

[65] Gabizon A, Amitay Y, Tzemach D, Gorin J, Shmeeda H, Zalipsky S. Therapeutic efficacy of a lipid-based prodrug of mitomycin C in pegylated liposomes: studies with human gastro-entero-pancreatic ectopic tumor models. Journal of Controlled Release 2012;160:245-53.

[66] Judson I, Radford JA, Harris M, Blay J-Y, Van Hoesel Q, Le Cesne A, et al. Randomised phase II trial of pegylated liposomal doxorubicin (DOXIL®/CAELYX®) versus doxorubicin in the treatment of advanced or metastatic soft tissue sarcoma: a study by the EORTC Soft Tissue and Bone Sarcoma Group. European Journal of Cancer 2001;37:870-7.

[67] Zhang L, Chan JM, Gu FX, Rhee J-W, Wang AZ, Radovic-Moreno AF, et al. self-assembled lipid− polymer hybrid nanoparticles: a robust drug delivery platform. ACS nano 2008;2:1696-702.

[68] Gordon S, Rabinowitz S. Macrophages as targets for drug delivery. Advanced Drug Delivery Reviews 1989;4:27-47.

[69] Van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. Journal of immunological methods 1996;193:93-9.

[70] Gao R, Stone WL, Huang T, Papas AM, Qui M. The uptake of tocopherols by RAW 264.7 macrophages. Nutrition journal 2002;1:2.

[71] Fukasawa M, Adachi H, Hirota K, Tsujimoto M, Arai H, Inoue K. SRB1, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells. Experimental cell research 1996;222:246-50.

[72] Schroit AJ, Fidler IJ. Effects of liposome structure and lipid composition on the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. Cancer research 1982;42:161-7.

[73] Allen T, Austin G, Chonn A, Lin L, Lee K. Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochimica et Biophysica Acta (BBA)-Biomembranes 1991;1061:56-64.

[74] Aramaki Y, Akiyama K, Hara T, Tsuchiya S. Recognition of charged liposomes by rat peritoneal and splenic macrophages: effects of fibronectin on the uptake of charged liposomes. European journal of pharmaceutical sciences 1995;3:63-70.

[75] Geelen T, Yeo SY, Paulis L, Starmans L, Nicolay K, Strijkers GJ. Internalization of paramagnetic phosphatidylserine-containing liposomes by macrophages. J Nanobiotechnol 2012;10:1-.

[76] Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nature Reviews Immunology 2013;13:621-34.

[77] Ribbons KA, Zhang X-J, Thompson JH, Greenberg SS, Moore WM, Kornmeier CM, et al. Potential role of nitric oxide in a model of chronic colitis in rhesus macaques. Gastroenterology 1995;108:705-11.

[78] Middleton S, Shorthouse M, Hunter J. Relaxation of distal colonic circular smooth muscle by nitric oxide derived from human leucocytes. Gut 1993;34:814-7.

[79] Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 1994;305:253-64.

[80] Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, et al. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 1996;111:871-85.

[81] Tötemeyer S, Sheppard M, Lloyd A, Roper D, Dowson C, Underhill D, et al. IFN-γ enhances production of nitric oxide from macrophages via a mechanism that depends on nucleotide oligomerization domain-2. The Journal of Immunology 2006;176:4804-10.

[82] Aramaki Y, Nitta F, Matsuno R, Morimura Y, Tsuchiya S. Inhibitory effects of negatively charged liposomes on nitric oxide production from macrophages stimulated by LPS. Biochemical and biophysical research communications 1996;220:1-6.

[83] Rogler G, Andus T. Cytokines in inflammatory bowel disease. World journal of surgery 1998;22:382-9.

[84] Carey R, Jurickova I, Ballard E, Bonkowski E, Han X, Xu H, et al. Activation of an IL-6: STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease. Inflammatory bowel diseases 2008;14:446-57.

[85] Fonseca de Oliveira B, Araujo Veloso C, Augusto Nogueira-Machado J, Martins Chaves M. High doses of in vitro beta-carotene, alpha-tocopherol and ascorbic acid induce

oxidative stress and secretion of IL-6 in peripheral blood mononuclear cells from healthy donors. Current aging science 2012;5:148-56.

[86] Lira FS, Rosa JC, Cunha CA, Ribeiro EB, do Nascimento CO, Oyama LM, et al. Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. Lipids Health Dis 2011;10:1186.

[87] Kanazawa S, Tsunoda T, Onuma E, Majima T, Kagiyama M, Kikuchi K. VEGF, basic-FGF, and TGF-β in Crohn's disease and ulcerative colitis: a novel mechanism of chronic intestinal inflammation. The American journal of gastroenterology 2001;96:822-8.

[88] Lawrance IC, Maxwell L, Doe W. Inflammation location, but not type, determines the increase in TGF-β1 and IGF-1 expression and collagen deposition in IBD intestine. Inflammatory bowel diseases 2001;7:16-26.

[89] Haller D, Holt L, Kim SC, Schwabe RF, Sartor RB, Jobin C. Transforming growth factor-β1 inhibits non-pathogenic gramnegative bacteria-induced NF-κB recruitment to the interleukin-6 gene promoter in intestinal epithelial cells through modulation of histone acetylation. Journal of Biological Chemistry 2003;278:23851-60.

[90] Zahangir Hosain M, Mori T, Kishimura A, Katayama Y. Synergy between phenotypic modulation and ROS neutralization in reduction of inflammatory response of hypoxic microglia by using phosphatidylserine and antioxidant containing liposomes. Journal of Biomaterials Science, Polymer Edition 2015:1-16.

CHAPTER 5

Conclusion Remarks

Mediators of inflammation induced by macrophages are critical for a variety of human inflammatory disorders. In most of the inflammatory diseases, once macrophages activated, it secretes mediators of inflammation such as cytokines, chemokine and causes an imbalance of such mediators. During the early and short inflammatory phase, macrophages exert pro-inflammatory functions but persistence of pro-inflammatory activity and altered function of macrophages result in the development of chronic inflammatory diseases such as neurological diseases, atopic dermatitis and ulcerative colitis.

Macrophages are highly plastic leukocytes that can enter various tissues under inflammatory or non-inflammatory conditions and assume different functional phenotypes according to the cues they receive from the environment. M1 macrophages (classically activated macrophages) are pro-inflammatory and have a central role in host defense against infection, while M2 macrophages (alternatively activated macrophages) are associated with responses to anti-inflammatory reactions and tissue remodeling. Transforming of macrophages into a proper phenotype to regulate the initiation, development, and ending of inflammatory diseases by targeting molecules in signal pathways and local microenvironment will be an attractive area for the therapies of various acute and chronic inflammatory diseases. In this thesis, I focused on the characters and inflammatory polarization of macrophage into anti-inflammatory phenotype to adapt to the microenvironment that might be a great promise for the treatment of inflammatory diseases.

CHAPTER 5

In chapter 2, I designed the preparation of a potential anti-inflammatory liposomes (PST-liposome), containing both PS and α-tocopherol and investigated their effects on anti-inflammatory response of hypoxia activated microglia, the residential macrophage of brain. These two components exhibited a clear synergistic anti-inflammatory effects which revealed by increased viability of activated microglia through reducing the production of both NO and inflammatory cytokines. The synergistic anti-inflammatory effects of PST-liposome indicates that dominant signalling pathways which are modulated either by PS recognition or ROS erasing with α-tocopherol will function independently to cause anti-inflammatory response in microglia. Thus, this PST-liposomes could be an effective modulator of neuroinflammation and, therefore, have clinical potential for microglial activation-mediated neurological diseases including those involving brain ischemia.

In chapter 3, I provided a polymer particle instead of liposome, because it expresses superior characteristics for *in vivo* application due to its stability. Thus, D, L-PLA micro-particle displaying PS on its surface was prepared and investigated its transforming function on macrophage. Actually, it suppress the production of pro-inflammatory cytokine showing the leading of M2 macrophage. Then, the particle was applied to the treatment of atopic dermatitis model mice. The particle successfully suppressed the inflammatory feature of the dermatitis indicating its potential usefulness for allergy treatment.

Therefore, in chapter 4, I proposed a biodegradable polymer-lipid hybrid nanoparticle (PST-PLNP) containing both anti-oxidants and anti-inflammatory molecule as an ideal formulation for decreasing the oxidative stress and inflammatory response produced by activated macrophages. To achieve the target, we incorporated α-tocopherol and phosphatidylserine in the nanoparticle and investigated their response in activated macrophages. These two components demonstrated a synergistic anti-inflammatory and

anti-oxidative effects by reducing the production of NO and inflammatory cytokine production in activated RAW.264.7 macrophages. Our data also demonstrated that PST-PLNP significantly increased viability and able to produce anti-inflammatory cytokine in activated macrophages. The potential anti-inflammatory and anti-oxidative effects of this particle indicates the inflammatory modulation of the macrophages. Therefore, this PST-PLNP could be a potential drug carrier and effective approach by targeting the macrophage phenotype for healing of chronic inflammatory diseases such as ulcerative colitis.

The novel approach that I have mentioned in this thesis may provide an extension of current inflammatory therapy. Although there still remain many obstacles and further investigation, the combination of anti-oxidant and ant-inflammatory property of the nanoparticle that I describe in this dissertation, might be a potential approach for the conversion of macrophage phenotype for the treatment of inflammatory diseases.

Accomplishments

List of Publications

1. Hosain, M.Z., Mori T, Kishimura A, Katayama Y. Synergy between phenotypic modulation and ROS neutralization in reduction of inflammatory response of hypoxic microglia by using phosphatidylserine and antioxidant containing liposomes. *Journal of Biomaterials Science, Polymer Edition* 2015:1-16.
2. Kumar, P., Hosain, M. Z., Kang, J. H., Takeo, M., Kishimura, A., Mori, T., & Katayama, Y."Suppression of atopic dermatitis in mice model by reducing inflammation utilizing phosphatidylserine-coated biodegradable microparticles." *Journal of Biomaterials Science, Polymer Edition* 26.18 (2015): 1465-1474.

List of Presentations

Oral presentation

Synergy of brain macrophages alteration and ROS erasing by using phosphatidylserine containing antioxidant liposomes toward brain ischemia therapy, 63rd SPSJ Annual meeting, May, 2014.

Poster presentation

Producing anti-inflammatory response to microglia using phosphatidylserine containing anti-oxidant liposome for reducing ischemic injury in brain, Japan Society for Analytical chemistry for young researchers in Kyushu, July, 2014.

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