

Development of nanoparticles for induction of immunotolerance towards the therapy of inflammation and allergic diseases

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<https://hdl.handle.net/2324/1959111>

出版情報 : Kyushu University, 2018, 博士 (工学) , 課程博士
バージョン :
権利関係 :



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Abstract

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Doctor of Philosophy

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2018

Thanks to the nanomedicine properties and characteristics that lipid-based nanoparticle and protein-based nanoparticle have, developing strategies to design tolerogenic nanoparticle have been extensively evolved. Whether encapsulation or conjugation of immunomodulatory molecules with nanoparticles is achieved through covalent, chemical, or physical interaction, the ultimate purposes are to improve efficacy of existing drugs and develop new therapeutic systems. Nanoparticle therapies are growing and used in the understanding of the underlying pathogenesis of immune-mediated diseases. Nanoparticles also have potential to restore peripheral immune tolerance or to regulate aberrant monocyte activities during severe inflammation. However, the functional outcome of nanoparticles depends on many intrinsic factors of nanoparticle and also is interfered by external factor such as route of administration. To address this issue and to utilize the potential of nanoparticle, development of an inert and simpler nanoparticle is required. In this thesis, I approached this goal through two methods.

In chapter 2, I could successfully prepare effective formulation of nanostructured lipid carrier (NLC) for delivery of $1,25(\text{OH})_2\text{D}_3$ to colonic inflammatory lesions via oral administration. NLC as lipid-based nanoparticle is suitable for incorporation of hydrophobic molecules. NLC orally delivered $1,25(\text{OH})_2\text{D}_3$ to the colon in mice and maintained a high concentration of $1,25(\text{OH})_2\text{D}_3$ in the colonic tissue for at least 12 h. The NLC showed multiple effects on the suppression of symptoms of colitis induced by dextran sodium sulfate, namely maintaining crypt structure, reducing the tissue concentration of inflammatory cytokines, suppressing the infiltration of polymorphonuclear leukocytes, and augmenting anti-inflammatory $\text{CX}_3\text{CR}_1^{\text{high}}$ macrophages. My NLCs containing $1,25(\text{OH})_2\text{D}_3$ may be an alternative treatment for IBD therapy.

In chapter 3, I followed first strategy which used NLC as carrier for regulation inflammation. I used same formulation of NLC for incorporation and delivery of all-trans retinoic acid (ATRA). NLC containing ATRA (NLC-RA) thus obtained was taken up by macrophages and induced anti-inflammatory response via suppressing NF- κ B signaling as well as via enhancing the production of anti-inflammatory cytokines. Moreover, NLC-RA enhanced differentiation of naïve T cells to regulatory T cells in the co-culture system with dendritic cells. These results suggest that NLC-RA is a promising alternative therapy for the autoimmune diseases especially intestinal bowel disease.

In chapter 4, I proposed a simple formulation of ovalbumin nanoparticles complexed with all-trans retinoic acid (OVA/RA NPs) induced phenotype of tolerogenic dendritic cells (tDCs). tDCs play a crucial role in the immunotolerance therapy because they induce anergic response for auto-reactive helper T cells and also enhance differentiation to regulatory T cells to maintain tolerance against auto-antigens. ATRA is one of the representative molecules to induce tDCs. As a result, OVA/RA NPs were taken up by DCs and successfully induced phenotype of tolerogenic dendritic cells (tDCs). These promising features of OVA/RA NPs will be suited for immunotolerance therapy and may be applicable to pulmonary delivery via inhalation to treat allergic asthma.

The novel approaches and findings in this research may provide new insights into the development of more straightforward methods to utilize the unique advantages of nanoparticles in immunotolerance therapy. And in a broader sense, they can lead realizing of more efficient and inexpensive medications for severe diseases.

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CHAPTER 1 Introduction

Vitamins are organic compounds (well known as micronutrient) which are synthesized by many bacteria, yeast, and plants, but not in mammals including humans [1]. Therefore, vitamins are required in trace amounts in the diet and/or commensal bacteria [2, 3]. Moreover, vitamins have important role on the development, maintenance of biological responses, and regulation of host immune system [1, 4, 5] for example in the regulation of gut immunity (Figure 1.1). Thus, deficient or inappropriate intake of vitamins frequently associates with the increased risk of infection, allergy, and inflammatory diseases [3, 4, 5].

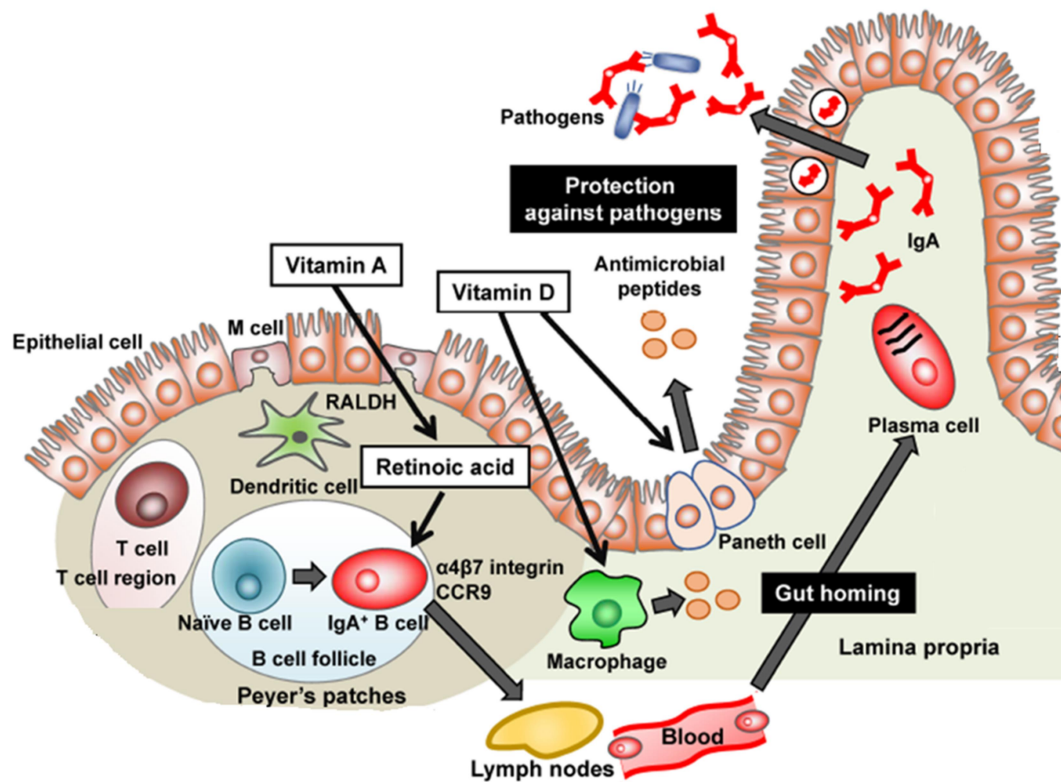


Figure 1.1. Various roles of vitamins in the regulation of gut immunity. Vitamin A is converted to retinoic acid by retinal dehydrogenases (RALDH) expressing dendritic cells (DCs) in the Peyer's patches, which induces the expression of gut-homing molecules ($\alpha 4 \beta 7$ integrin and CCR9) on antigen-primed cells (e.g., IgA⁺ B cells) and allows them to traffic into the intestinal lamina propria. In the lamina propria, IgA⁺ B cells differentiate into IgA producing plasma cells. IgA is then transported into the intestinal lumen, where it binds to pathogens to inhibit their invasion and function. Vitamin D enhances production of antimicrobial peptides from Paneth cells and macrophages via vitamin D receptor, which provides an additional immunosurveillance system [5].

Recently, vitamins A and D have received much attention because they have been shown to have an unexpected and crucial effect on the immune response, such as lymphocyte activation and proliferation, helper T (Th) cell differentiation, tissue-specific lymphocyte homing, the production of specific antibody isotypes and regulation of the immune response [5]. A metabolite of vitamin A and D, retinoic acid and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂VD₃) respectively, also have clinical potential for modulating tissue-specific immune responses and for preventing and/or treating inflammation and autoimmunity in highly specific ways [6].

1.1 Vitamin D₃

Vitamin D₃ (VD₃), physiological form of vitamin D, is synthesized from cholesterol in the skin during the exposure of sunlight [7], specifically ultraviolet B radiation (wavelengths of 270-300 nm). VD₃ is then converted in the liver to 25-dihydroxyvitamin D₃ (25(OH)VD₃), which is the main circulating form of VD₃. Finally, 25(OH)VD₃ is metabolized in the kidneys and/or cells of the immune system to 1,25(OH)₂VD₃, the most physiologically active VD₃ metabolite (Figure 1.2) [6, 8]. 1,25(OH)₂VD₃ is a ligand of vitamin D receptor (VDR) and facilitates intestinal absorption of calcium, which is responsible for the appropriate remodeling of bone [9].

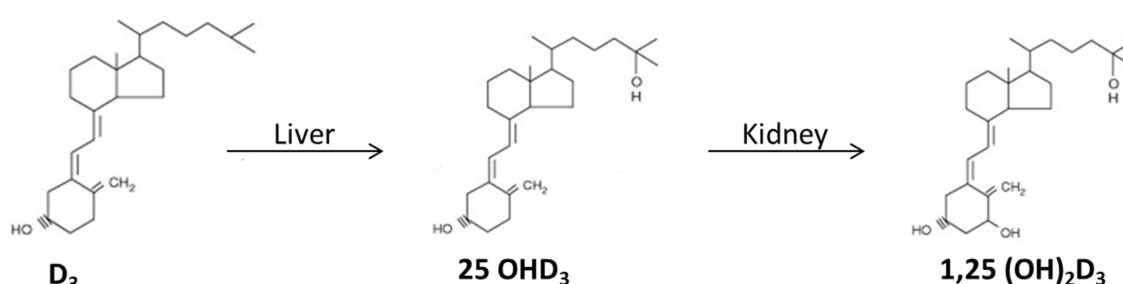


Figure 1.2. Vitamin D₃ metabolism. The 25-hydroxylation occurs primarily in the liver, although other tissues have the requisite enzymatic activity. The major 25-hydroxylases in the liver include the mitochondrial enzyme CYP27A1 and the microsomal enzyme CYP2R1 (among others). 25OHD₃ is then converted to its active form 1,25(OH)₂D₃ primarily in the kidney by the enzyme CYP27B1. As for the 25-hydroxylases, numerous tissues in addition to the kidney express CYP27B1 [10].

1,25(OH)₂VD₃ enhances the production of antibacterial peptides from intestinal Paneth cells and macrophages (Figure 1.1), which provide innate type anti-microbial immunity [11, 12]. 1,25(OH)₂VD₃ also work on strengthens the epithelial tight junction in the gut by upregulating tight junction proteins [12] and prevent differentiation and maturation of DCs (Figure 1.3) [5, 13]. Insufficiency of 1,25(OH)₂VD₃ has been reported to be linked to many diseases, including Crohn's disease (CD), ulcerative colitis (UC), asthma, diabetes, atopic dermatitis (AD), autoimmunity, and metabolic disease [14-18].

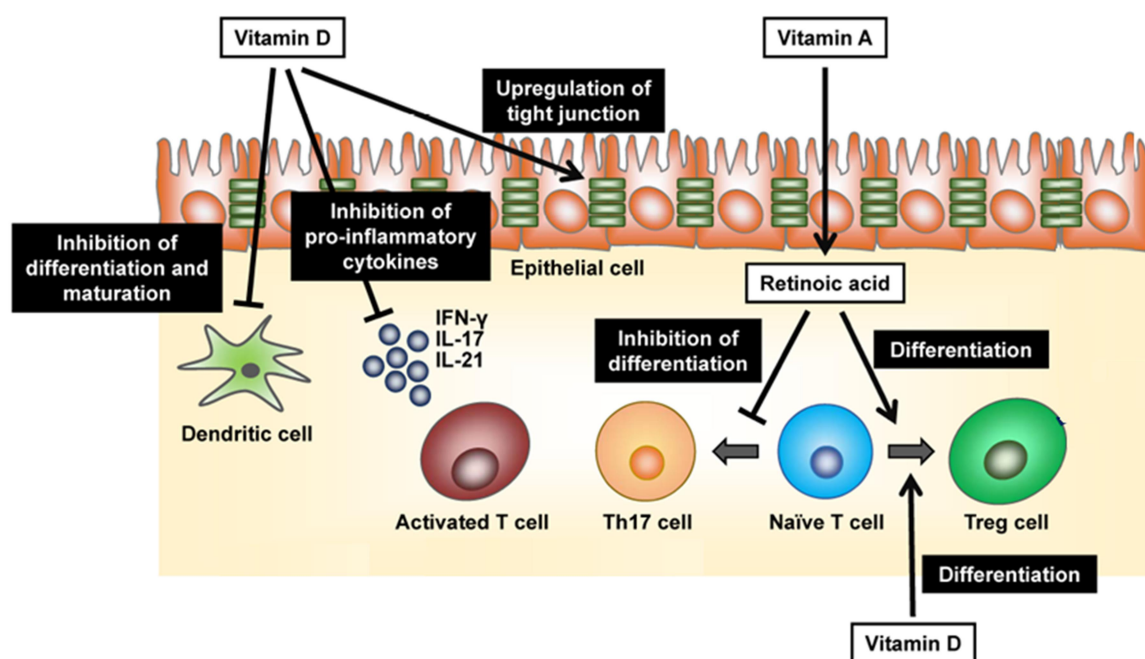


Figure 1.3. Role of vitamin D and A in the maintenance of immune homeostasis in the gut. Vitamin A-derived retinoic acid promotes the differentiation of naïve T cells to regulatory T (Treg) cells and simultaneously inhibits the induction of Th17 cells in the steady state. Like retinoic acid, Vitamin D (as an active form 1 α ,25-dihydroxyvitamin D₃) inhibits the production of pro-inflammatory cytokines such as IFN- γ , IL-17, and IL-21 from T cells together with the promoted differentiation of Treg cells. It also prevents differentiation and maturation of DCs and increases the expression of tight junction protein such as claudins in the epithelial cells [5].

1.2 Retinoic acid

Vitamin A can be obtained from plant or animal-derived food in pro-vitamin A carotenoid or β -carotene form, which is water soluble and not stored in the body. Conversion of β -carotene to vitamin A requires oxidation by members of the alcohol dehydrogenase (ADH) family or retinol dehydrogenase (RDH) family to be converted into retinal, which is reversibly reduced to retinol, enabling uptake by cells. Epithelial cells and dendritic cells (DCs) express retinal dehydrogenase (RALDH), expressed in generate all-trans-retinoic acid (Figure 1.4) [19]. Using these pathways, the human body can make all the required vitamin A from plant-derived carotenoids.

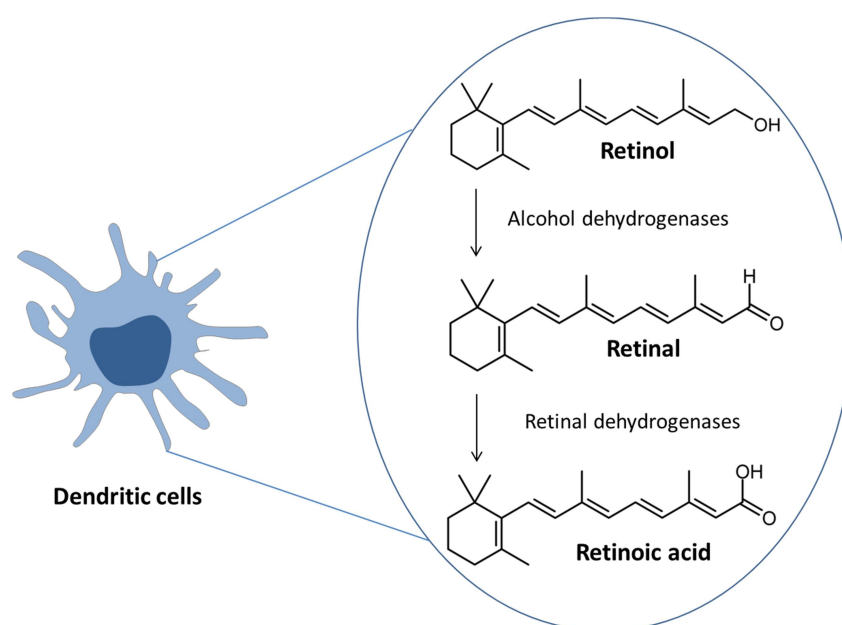


Figure 1.4. Vitamin A metabolism. In dendritic cells, retinol undergoes a series of oxidation steps catalyzed by alcohol dehydrogenases or short chain dehydrogenase reductases and retinal dehydrogenases, to produce retinal and the most biologically active metabolite; retinoic acid, respectively.

Retinoic acid (RA) is the active form of vitamin A, which can directly influence naïve T cell (Figure 1.3) [20-22]. Differentiation of regulatory T (Treg) cells is favored over Th17 cell polarization in the presence of retinoic acid [20-23] by inhibiting IL-6 receptor expression [22, 24]. Moreover, RA induces expression of Foxp3 in naïve T cells also through retinoic acid receptors (RAR) and thus enhances the preferential induction of Treg cells.

1.3 Nanoparticle-based immune modulation

The application of nanotechnology for clinical, is well known as nanomedicine, nanoparticle, or nanocarrier, which has many potentials to improve health care. Nanotechnology could be used in imaging, early diagnosis, pathological tissue analysis, and especially in drug delivery. Moreover, nanomedicine can allow not only the development of new therapeutic agents but also the improvement in the efficacy of existing drugs [25-28]. Focus on application of nanomedicine in immune regulation, the number of nanoparticle therapies is growing, fueled by innovations in nanotechnology and advances in the understanding of the underlying pathogenesis of immune-mediated diseases. Nanoparticles may be engineered for the purposes of restoring peripheral immune tolerance or to regulate aberrant monocyte activities during severe inflammation by the specific target which could interact with the mononuclear phagocyte system [29-33].

The functional outcome of nanoparticle immune modulation depends on numerous factors that are intrinsic nanoparticles, such as material, size, shape, and charge [34, 35], as well as extrinsic factors such as route of administration [29, 36]. Nanoparticles have been designed to deliver anti-inflammatory cargos to target tissue leading to reductions in the systemic therapeutic dose and side effects. Moreover, nanoparticles can be used for delivering cargos to antigen presenting cells (dendritic cells and macrophages) for modulation of antigen presentation and downregulate innate immune signals and may activate regulatory adaptive immune system [31, 33, 37]. All strategy of nanotechnology-based treatment depending on their cargo is summarized in Figure 1.5.

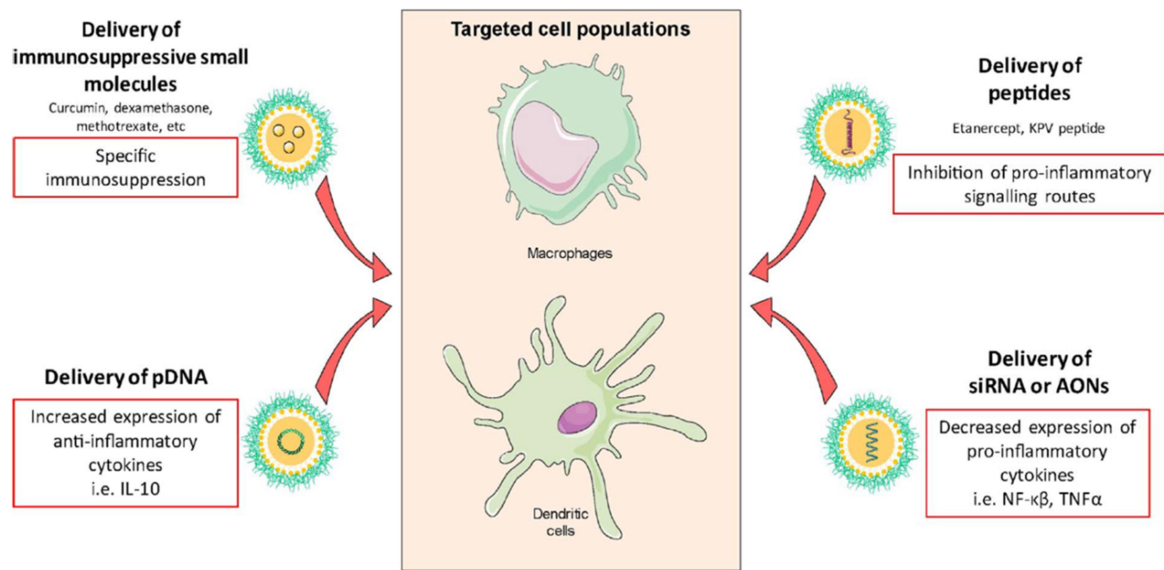


Figure 1.5. First, the delivery of small immunomodulatory molecules has been extensively explored in the suppression of the inflammatory activity of macrophages and dendritic cells (DCs). Second, the delivery of anti-inflammatory peptides has been used in different pathologies. Finally, gene therapy strategies include: pDNA delivery for the expression of anti-inflammatory cytokines, and siRNA or antisense oligonucleotide (AONs) delivery for the downregulation of pro-inflammatory molecules expression [37].

1.4 Overview of this thesis

In the path to utilize the potential of nanoparticle as an inert and tolerogenic nanoparticle, I approached this goal through two methods.

In the first approach, I tried to enhance suppressive effect on inflammation of endogenous vitamin D₃ by the by preparing effective formulation of nanostructured lipid carrier (NLC). NLC as lipid-based nanoparticle is suitable for incorporation of hydrophobic molecules. NLC orally delivered 1,25(OH)₂D₃ to the colon in mice and maintained a high concentration of 1,25(OH)₂D₃ in the colonic tissue for at least 12 h. The NLC showed multiple effects on the suppression of symptoms of colitis induced by dextran sodium sulfate, namely maintaining crypt structure, reducing the tissue concentration of inflammatory cytokines, suppressing the infiltration of polymorphonuclear leukocytes, and augmenting anti-inflammatory CX₃CR1^{high} macrophages. My NLCs containing 1,25(OH)₂D₃ may be an alternative treatment for IBD therapy. Moreover, I also utilized NLC as carrier for another vitamin (retinoic acid). Linear with NLCs containing 1,25(OH)₂D₃ result, NLC containing retinoic acid suppressed inflammation and also has potential recruit adaptive immune cellular by enhancement of Treg cell differentiation.

In the second approach, I proposed a simple formulation of ovalbumin nanoparticles complexed with all-trans retinoic acid (OVA/RA NPs) induced phenotype of tolerogenic dendritic cells (tDCs). tDCs play a crucial role in the immunotolerance therapy because they induce anergic response for auto-reactive helper T cells and also enhance differentiation to regulatory T cells to maintain tolerance against auto-antigens. All-trans retinoic acid (ATRA) is one of the representative molecules to induce tDCs. As a result, OVA/RA NPs were taken up by DCs and successfully induced phenotype of tolerogenic dendritic cells (tDCs). These promising features of OVA/RA NPs will be suited for immunotolerance therapy and may be applicable to pulmonary delivery via inhalation to treat allergic asthma.

Thereby, finding new and straightforward methods to utilize the unique advantages of nanoparticles in immunotolerance therapy can be promising to realize more efficient and inexpensive medications for severe diseases.

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CHAPTER 2

Therapeutic effect of vitamin D₃-containing nanostructured lipid carriers on inflammatory bowel disease

2.1 Introduction

Inflammatory bowel disease (IBD) is a group of chronic inflammatory conditions in the gastrointestinal (GI) tract that leads to the destruction of the epithelial barrier in the small and large bowel [1]. Crohn's disease (CD) and ulcerative colitis (UC) are types of major IBD. IBD patients show accumulation of immune cells in the GI tract [1-4], which induces the production of proinflammatory cytokines and reactive oxygen species (ROS) [5]. Such inflammatory response causes further dysregulation of the mucosal immune system as well as the destruction of the epithelial barrier [6]. In the current regimens of IBD therapy, steroids and anti-inflammatory drugs such as 5-aminosalicylic acid are used to control the acute inflammation of IBD [2]. In the refractory case, immunosuppressant drugs and tumor necrosis factor- α (TNF- α) antagonists are used [2]. However, these current regimens for IBD are not always successful [4]. Thus, there continues to be a medical need for an effective and safe therapy for IBD [7].

Vitamin D₃ is a pleiotropic hormone that conventionally controls calcium homeostasis and bone mineralization [8]. Recently, a modulatory role of vitamin D₃ on the activity of immune cells has received attention [9, 10]. Vitamin D₃ is absorbed from the diet and synthesized in the skin during sun exposure. Vitamin D₃ is converted to 25(OH)D₃ and then 1,25(OH)₂D₃ by different enzymes existing in many kinds of cells, especially liver, kidney, and immune cells [8-10]. 1,25(OH)₂D₃, which is the active form of vitamin D₃, is an agonist of the vitamin D₃ receptor (VDR). VDR is a nuclear receptor that activates the expression of target genes in various kinds of cells [8-10]. Because patients with IBD, especially those with CD, often show a deficiency of 25(OH)D₃ and 1,25(OH)₂D₃ in their serum, a vitamin D₃ deficiency has been suspected to play a key role in the pathogenesis and progression of IBD [11, 12]. Recent experiments based on *in vitro* and IBD model animals have revealed that vitamin D₃ contributes to suppressing the progression of IBD through multiple pathways including: (1) suppression of both innate and acquired immunity by direct and cytokine-mediated regulation of immune cells [9, 10, 13], (2) mucosal barrier healing by influencing

the intestinal epithelium [14, 15], and (3) maintaining the balance of the gut microbiome by activating secretion of antimicrobial peptides from macrophages and Paneth cells [16-18].

The multiple therapeutic functions of vitamin D₃ toward IBD are advantageous compared with the presently available drugs that work to regulate the inflammatory response of immune cells. Thus, vitamin D₃ has received significant attention for the therapy of IBD [19-21]. Three clinical trials for CD patients have been conducted so far. These trials showed that oral administration of vitamin D₃ or its prodrug improved symptoms of CD [22-24] and tended to reduce the relapse rate [24]. However, the results from one of the clinical trials indicated that to improve the symptoms, a dosage of vitamin D₃ much higher than the conventionally accepted dosage was required [23]. A high dosage of vitamin D₃ is known to produce adverse effects such as hypercalcemia [20]. Thus, effective delivery of vitamin D₃ to inflammatory lesions of the GI tract should be developed so that the dosage can be reduced. Three pioneering works have reported the effective delivery of vitamin D₃ to the inflammatory lesions of IBD model mice. Froicu and Cantorna reported that rectal administration of 1,25(OH)₂D₃ showed a better therapeutic effect than oral administration because the rectal administration can directly deliver 1,25(OH)₂D₃ to the inflammatory lesions [25]. Laverny et al. also studied the advantages of rectal administration. They found a beneficial effect from the rectal administration of a vitamin D₃ analogue on an IBD model without inducing hypercalcemia [26]. Compared with rectal administration, oral administration is preferred because of the improved accessibility to the ileum where inflammation occurs in CD [2]. To enable oral administration, Groff et al. synthesized β -glucuronide derivatives of vitamin D₃ [27]. These hydrophilic derivatives were designed to not be absorbed in the upper GI tract and instead be absorbed in the ileum and colon after hydrolysis of the β -glucuronyl group by enzymes produced from intestinal bacteria.

Recently, nano-sized particles have received attention as oral delivery carriers used to target inflammatory tissue in IBD [28, 29]. One of the most successful oral delivery carriers is nanostructured lipid carriers (NLCs) [30-32]. Herein, I tried to develop NLCs to deliver 1,25(OH)₂D₃ to inflammatory lesions by oral administration. NLCs, which were originally developed by Müller, are composed of a solid lipid core of acylglycerols mixed with a small amount of oil compound that works to raise the loading capacity of hydrophobic drugs [30-32]. NLCs are stable in gastric fluid, but are gradually degraded in the intestine by lipase, which enhances the absorption of drugs in the intestine [31]. In general, preparation of NLCs requires a temperature high enough (typically ~80 °C) to melt solid lipids to enable their emulsification [30-32]. However, 1,25(OH)₂D₃ is unstable at high temperatures [33, 34].

Ultrasonication, which is applied to tune the size of the NLCs during preparation, is known to generate radical species [35] that can also damage 1,25(OH)₂D₃. To avoid the destruction of 1,25(OH)₂D₃ during NLC preparation, I developed a new fabrication procedure for NLCs containing 1,25(OH)₂D₃. The NLCs thus obtained were orally administered to mice. We found that the NLCs maintained a high concentration of 1,25(OH)₂D₃ in colonic tissue for a relatively long time and significantly suppressed the progression of disease in IBD model mice.

2.2 Results and discussion

2.2.1 NLC preparation

NLCs are generally prepared at high temperature to melt the solid lipid components. At the high temperature, pre-emulsions are prepared by high speed stirring, and then their size is reduced by ultrasonication or high pressure homogenization [30-32]. Because vitamin D₃ is sensitive to high temperature and reactive to radical species generated by ultrasonication [33, 34], the conventional NLC preparation conditions are not suitable. To avoid high temperature, we decided to use organic solvent to dissolve the solid lipid and 1,25(OH)₂D₃ to prepare the pre-emulsion at ambient temperature. As the core components of the NLCs, I selected glycerol monostearate (GMS) and α -tocopherol as the solid and liquid lipid, respectively. α -Tocopherol is a representative antioxidant and is expected to protect 1,25(OH)₂D₃ from the attack of radical species generated by ultrasonication. α -Tocopherol also functions to suppress inflammation by neutralizing the ROS [40,41]. To further protect 1,25(OH)₂D₃ from radical species, I added ascorbic acid in the aqueous phase [42]. As surfactants to stabilize the NLC, a combination of a neutral polymer (Pluronic F127) and phospholipids (phosphatidylcholine (PC):phosphatidylserine (PS) = 4:1) was selected. Negatively charged PS was mixed with the phospholipids because negative particles were reported to adhere to inflamed colitis tissue more than cationic and neutral particles [43]. PS may also help to target residential macrophages in inflammatory lesions because macrophages possess specific receptors for PS [44, 45]. All the selected components of the NLCs are approved materials for oral administration.

Table 2.1 summarizes the characteristics of the NLCs prepared under different conditions. High speed stirring alone provided large NLCs (~270 nm) with a wide size

distribution (run 1). Because a particle size of about 100 nm is reported to be suitable for accumulation in inflamed colonic tissue [46, 47], ultrasonication was required to reduce the size of the NLCs. Ultrasonication alone at 30% power provided NLCs of ~100 nm (run 4). However, the content of intact 1,25(OH)₂D₃ in the NLCs was quite low, probably because of the destruction by radical species. The combination of high speed stirring and lower-power ultrasonication provided ~100 nm NLCs while minimizing destruction of 1,25(OH)₂D₃ (run 5). The obtained NLCs were confirmed to be a spherical shape by TEM (Figure 2.1A). I concluded that the preparation condition used for run 5 was the best among the ones I examined. Without ascorbic acid in the aqueous phase, the content of the intact 1,25(OH)₂D₃ in the NLC-D3 decreased to 1/3 of that from run 5 (data not shown), confirming the crucial role of ascorbic acid in protecting 1,25(OH)₂D₃ from destruction by radical species. A dispersion of NLC-D3 could be stored for at least 45 days without causing changes in its size and size distribution (Figure 2.1B). Table 2.2 summarizes the NLCs prepared by the established condition. The content of the NLCs was varied as specified, but the size and ζ -potential of the NLCs were consistent except for the NLCs without PS [NLC-DiO (-) PS]. The larger size and less negative ζ -potential of these NLCs were ascribed to the absence of negatively charged PS on the surface.

Table 2.1. Characteristics of NLC-D3 prepared under various conditions. Data are expressed as mean $\pm$ SD ($n = 3$).

run	condition		size (nm)	polydispersity index	1,25(OH) ₂ D ₃ in NLC (μ g)	1,25(OH) ₂ D ₃ in aq. phase (μ g)	entrapment efficacy (%)
	homogenization	ultrasonication					
1	15 min	-	274 $\pm$ 28	0.74 $\pm$ 0.12	12.53 $\pm$ 0.55	3.31 $\pm$ 0.49	62.7 $\pm$ 2.7
2 ^a	-	10%, 5 min	-	-	-	-	-
3	-	10%, 15 min	597 $\pm$ 128	0.72 $\pm$ 0.16	2.66 $\pm$ 0.41	0.73 $\pm$ 0.64	13.3 $\pm$ 2.1
4	-	30%, 5 min	116 $\pm$ 2	0.33 $\pm$ 0.01	5.76 $\pm$ 0.88	0.27 $\pm$ 0.24	28.8 $\pm$ 4.4
5	15 min	10%, 5 min	110 $\pm$ 4	0.23 $\pm$ 0.01	12.57 $\pm$ 0.64	1.32 $\pm$ 0.60	62.8 $\pm$ 3.2

^a Stable NLC dispersion was not obtained (NLC was aggregated after evaporation of organic solvent).

Table 2. 2. Characteristics of NLCs in 10mM HEPES (pH 7.4). Data are expressed as mean $\pm$ SD ($n = 3$).

name	content	size (nm)	polydispersity index	ζ -potential (mV)
NLC	-	108 $\pm$ 6	0.23 $\pm$ 0.02	-17.60 $\pm$ 0.96
NLC-D3	1,25(OH) ₂ D ₃	110 $\pm$ 4	0.23 $\pm$ 0.01	-17.10 $\pm$ 0.30
NLC-DiO	DiO	106 $\pm$ 7	0.24 $\pm$ 0.00	-16.70 $\pm$ 0.90
NLC-DiO (-) PS ^a	DiO	171 $\pm$ 5	0.24 $\pm$ 0.00	-9.94 $\pm$ 0.37

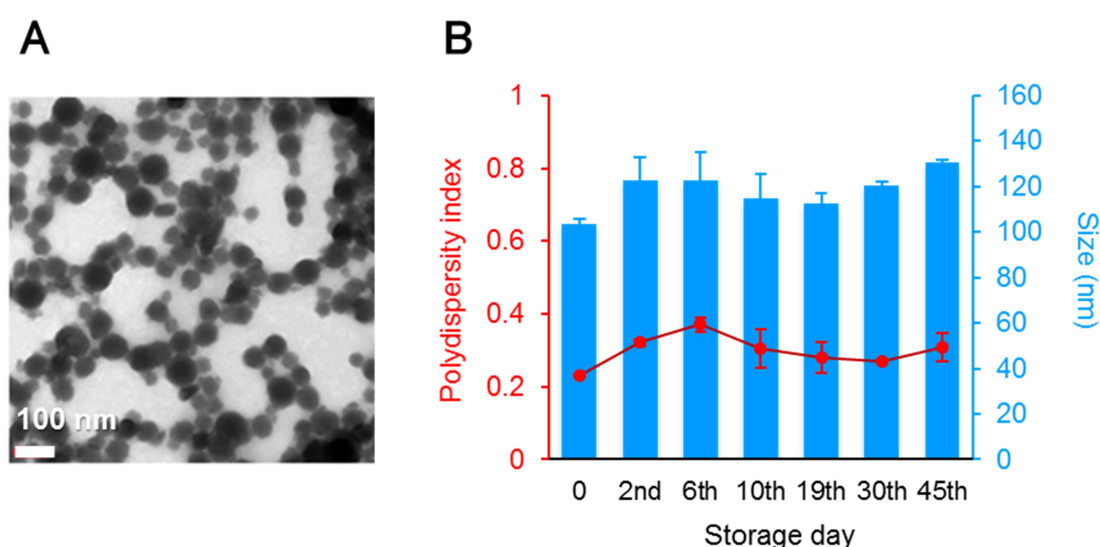


Figure 2.1. TEM image of NLC-D3 (A) and effect of storage time of NLC-D3 dispersion at 5 °C on its size and size distribution (B).

2.2.2 Cellular uptake and cytotoxicity of NLCs toward macrophages

I evaluated the cellular uptake and cytotoxicity of NLCs against RAW 264.7 macrophages. Macrophages have been reported to significantly contribute to the progression of the inflammatory reaction in IBD [48, 49]. As shown in Figure 2.2A, NLC-DiO (0.01%) was actively taken up by the macrophages after 24 h incubation. Treatment of NLCs without PS resulted in a significant reduction in the cellular uptake (Figure 2.2B), showing the marked effect of PS on the recognition by the macrophages. PS on the NLC surface may

contribute to the specific delivery of NLCs to residential macrophages in inflammatory lesions *in vivo*. Despite the active cellular uptake of the NLCs, neither the NLCs nor NLC-D3 showed toxicity toward the macrophages up to a concentration of 0.05 % (Figure 2.2C).

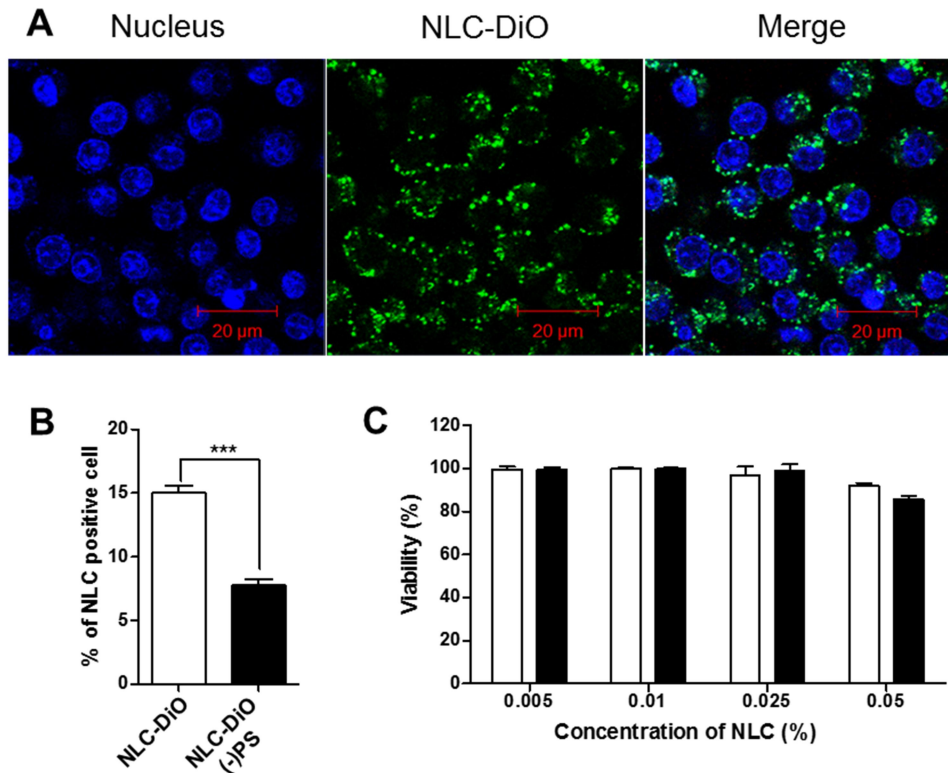


Figure 2.2. (A) Cellular uptake of NLCs by RAW 264.7 macrophages after 24 h incubation. Blue and green colors represent the nucleus and NLC-DiO, respectively. (B) Cellular uptake of NLCs and PS-removed NLCs after 6 h incubation. (C) Viability of RAW 264.7 macrophages after treatment with empty NLCs (white bar) and NLC-D3 (filled bar) for 24 h. Macrophages were treated with each NLC (0.01%) for 6 h (B) or 24 h (A, C) at 37°C. Data in B and C represent the mean $\pm$ SD (n = 3). *** P < 0.001.

2.2.3 Anti-inflammatory effect of NLC-D3 on macrophages

The inflammatory response of immune cells against LPS derived from intestinal bacteria contributes to the progression of IBD [50, 51]. LPS binds to TLR4, which induces the inflammatory response mainly via NF- κ B dependent pathway [52]. Because 1,25(OH) $_2$ D $_3$ has been reported to suppress the inflammatory response in macrophages [53-55], I examined the suppressive effect of NLC-D3 in activation of the NF- κ B pathway triggered by LPS. To

this end, I took advantage of the RAW 264.7 cell line transfected with secreted alkaline phosphatase (SEAP) as a reporter gene, which is under the transcriptional control of an NF- κ B response element. As shown in Figure 2.3A, not only NLC-D3 but also empty NLCs significantly suppressed the activation of the NF- κ B pathway, indicating that some components of the empty NLCs have an anti-inflammatory effect. To clarify the contribution of each component to the suppression of the NF- κ B pathway, I prepared various empty NLCs lacking each component. As shown in Fig. 2B, NLCs lacking PS ((-) PS) did not affect the anti-inflammatory effect, while NLCs lacking α -tocopherol ((-) Toco) diminished the anti-inflammatory effect. Thus, the suppression of the NF- κ B pathway by the empty NLCs resulted from the neutralization of ROS by α -tocopherol [40, 41]. Hereafter, I used NLC-D3 containing both PS and α -tocopherol.

Next I assessed the suppressive effect of NLC-D3 on the expression of proinflammatory cytokines in response to the LPS stimulation. As shown in Fig. 2C, the treatment with NLC-D3 more potently downregulated the expression of all three cytokines (IL-6, TNF- α , and IL-1 β) compared to the empty NLCs. Regarding suppression of inflammation, vitamin D inhibits production of pro-inflammatory cytokines not only via direct inhibition of NF- κ B by its receptor complex VDR/RXR, but also via the inhibition of p38 MAP kinase which have been identified as central signal transducers of inflammatory signals [56]. This result underscored the suppressive effect of 1,25(OH) $_2$ D $_3$ on the inflammatory response of the macrophages.

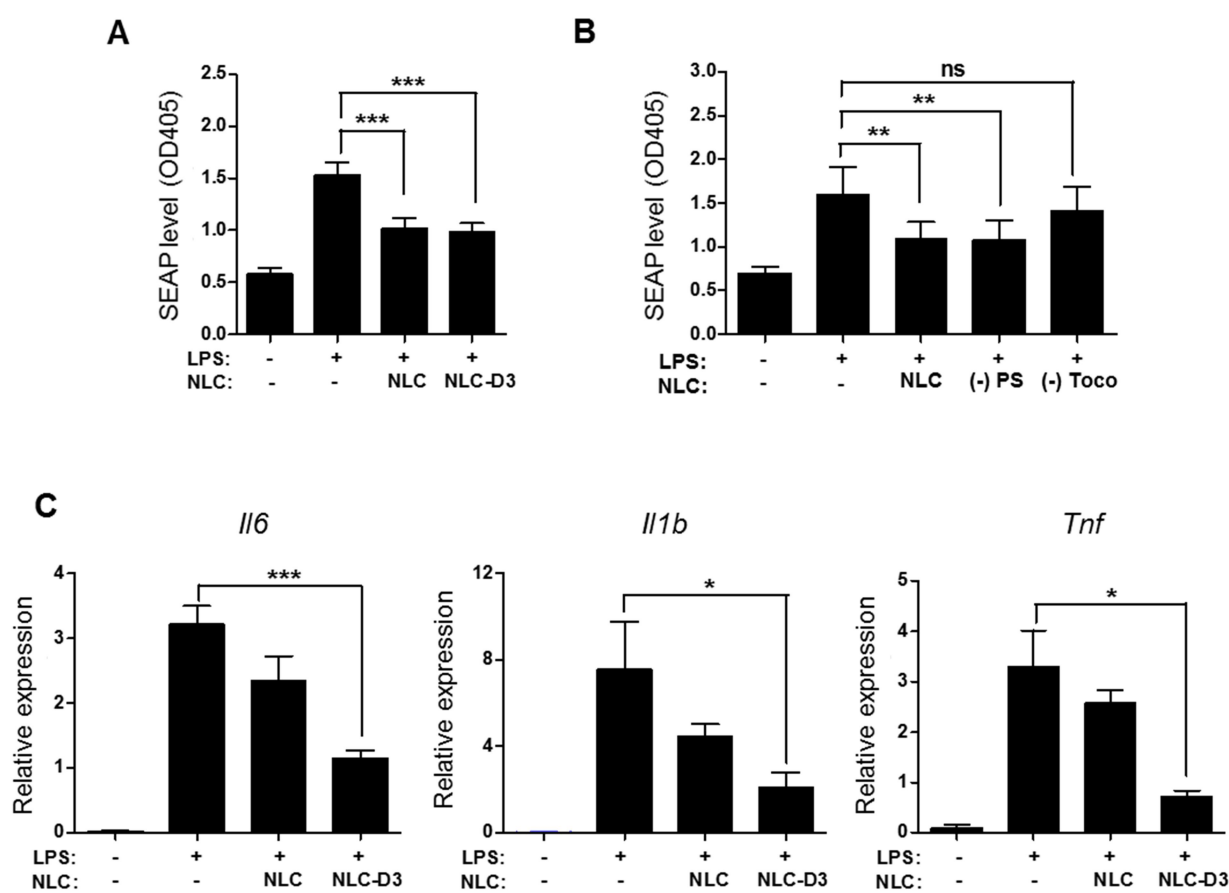


Figure 2.3. (A, B) Suppressive effects of NLCs on the LPS-induced NF- κ B activation in RAW264.7 macrophages. After addition of each NLC [0.01%, containing 8.49 nM of 1,25(OH) $_2$ D $_3$] to macrophages for 6 h, LPS (20 ng/mL) was added and incubated for a further 18 h. Absorbance resulting from the substrate reacted with SEAP was measured at 405 nm. (-) PS and (-) Toco in B indicate NLC-D3 prepared without PS and α -tocopherol, respectively. (C) Suppressive effect of NLCs on the gene expression of proinflammatory cytokines in RAW264.7 macrophages. After similarly treating the macrophages with A and B, gene expression was evaluated by qRT-PCR. Data in A–C are the mean $\pm$ SD ($n = 3$). N.S., not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2.2.4 NLC facilitated delivery of vitamin D3 to the colon

To evaluate the delivery efficiency of 1,25(OH) $_2$ D $_3$ into the colon by the NLC-D3, I measured the concentration of 1,25(OH) $_2$ D $_3$ in the colonic tissue after gavage administration. As shown in Figure 2.4, administration of 1,25(OH) $_2$ D $_3$ as a saline dispersion failed to increase 1,25(OH) $_2$ D $_3$ concentrations in the colon tissue. In contrast, NLC-D3 significantly

raised the colonic concentration of $1,25(\text{OH})_2\text{D}_3$ up to 3-fold compared with the basal level and this high level persisted at least 12 h after oral administration. Thus, the encapsulation of $1,25(\text{OH})_2\text{D}_3$ in NLC allowed sustained delivery of $1,25(\text{OH})_2\text{D}_3$ to the colon for a long duration.

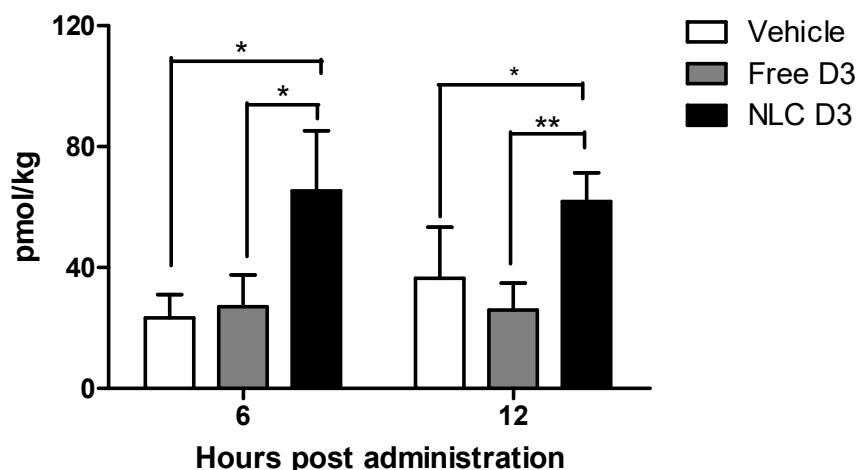


Figure 2.4. $1,25(\text{OH})_2\text{D}_3$ levels in colonic tissues after oral administration of vehicle, free-D3 or NLC-D3. Data are presented as the mean $\pm$ SD (n = 3). *P < 0.05 and **P < 0.01

2.2.5 NLC-D3 ameliorated symptoms of DSS-induced colitis

I subsequently assessed the therapeutic effect of NLC-D3 on DSS-induced colitis, which has been widely utilized as IBD model [56]. The clinical score was monitored on the basis of the severity of diarrhea and colonic hemorrhage [57]. A dose of ca. 120 pmol/day of $1,25(\text{OH})_2\text{D}_3$ was chosen for this study, because this dose was confirmed not to induce hypercalcemia in previous studies [25, 27]. In the vehicle- or free-D3-treated mice, the clinical symptoms increased gradually and culminated around Day 5 (Figure 2.5A). Treatment with NLC-D3 ameliorated the clinical symptoms from Day 3 onward, and decrease in body weight was suppressed from Day 6 (Fig. 2.5 B). As shown in Fig. 4C, histological examination of the colonic tissue from the vehicle and free-D3 groups showed severe inflammatory symptoms, namely infiltration of immune cells, crypt destruction, and loss and disordering of goblet cells which are shown as light blue color by Alcian blue

staining. In accordance with the clinical score, these colonic inflammatory symptoms were also significantly suppressed in the NLC-D3 group (Fig. 2.5 C, D). In spite of the therapeutic effect on the colonic tissue, NLC-D3 failed to suppress the shortening of colon length, which is generally used as an index to measure the severity of inflammation (Fig. S3).

Pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , mediate development of IBD [5] as well as DSS-induced colitis [58], I observed that expression of *Il6* was remarkably downregulated in the colonic tissue of mice treated with NLC-D3 but not free-D3 (Figure 2.5D). The expression level of *Il1b* was less than half in the NLC-D3 group compared with the vehicle group although the difference was not statistically significant. There was no obvious difference in expression of *Tnf* among the groups. Administration of DSS causes not only colitis but also systemic inflammation that is characterized by splenomegaly owing to bacterial translocation from colonic lamina propria [57]. Treatment with NLC-D3 also prevented splenomegaly, suggesting attenuation of bacterial translocation (Figure 2.5E, F). In contrast, treatment with free-D3 failed to protect the development of splenomegaly. Taken together, these results demonstrate that administration of NLC-D3 alleviates both local and systemic inflammation induced by DSS.

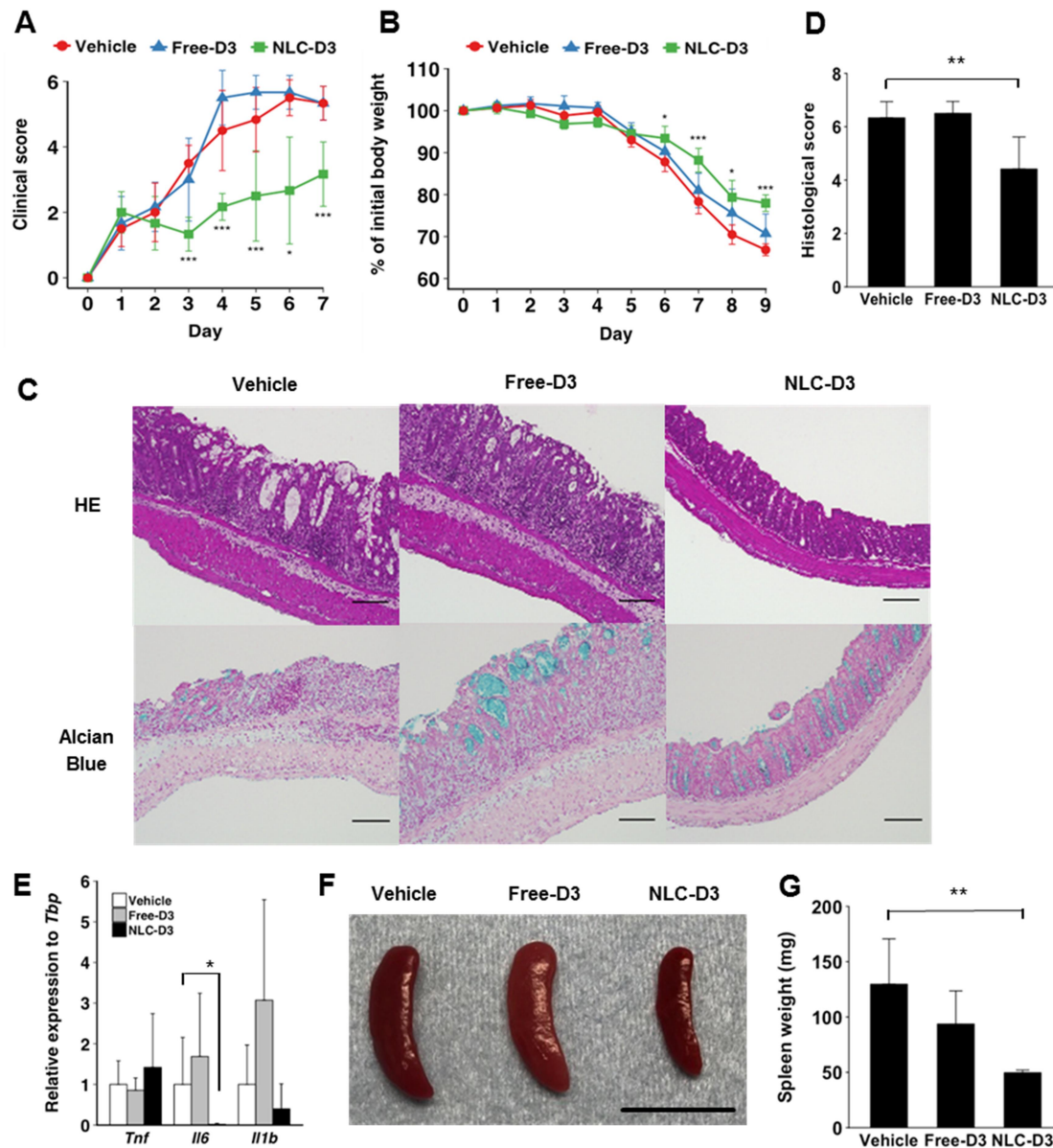


Figure 2.5. Effects of NLC-D3 on dextran sodium sulfate-induced colitis. (A-G) Mice were administered 2% dextran sodium sulfate (DSS) for 6 days followed by normal drinking water. During the course of the experiment, the mice received oral gavage of the vehicle, free-D3, or NLC-D3 once a day from Day 0. The clinical score of colitis (A) and body weight (B) were monitored. Sections of the colonic tissues on Day 7 were subjected to hematoxylin and eosin staining (C, upper panels) and Alcian blue staining to detect goblet cells (C, lower panels). Scale bars: 100 μ m. The histological score was calculated based on the criteria described in the Materials and Methods section (D). Expression levels of proinflammatory cytokines in the colonic tissue on Day 7 are shown (E). The data were normalized to the expression of *Tbp*. Representative images of the spleen in each group (F) and the splenic weight (G) on Day 7 are shown. Scale bars: 1 cm. Data are presented as the mean $\pm$ SD (n = 6). *P < 0.05, **P < 0.01 vs vehicle control. Data are representative of two independent experiments.

2.2.6 NLC-D3 altered the composition of the immune cells in the colonic lamina propria

To further delineate the effect of NLC-D3 treatment on DSS-induced colitis, I analyzed the composition of the immune cells in the colonic lamina propria by flow cytometry. I found that administration of NLC-D3 diminished infiltration of polymorphonuclear leukocytes (PMN), namely eosinophils and neutrophils (Figure 2.6A, B), whereas NLC-D3 treatment did not affect the frequency of the monocytes (Figure 2.6C). NLC-D3 treatment slightly increased the frequency of CD11b⁺Ly6C⁻Ly6G⁻MHC class II⁺ total macrophages (CD11b⁺MHC class II⁺) although the difference was not statistically significant (Figure 2.6D). Among the macrophage populations in the gut, the CX₃CR1^{high} subset shows anti-inflammatory properties [59] and prevents the translocation of bacteria by phagocytosis [60]. This macrophage subset was markedly increased in NLC-D3-treated mice compared with vehicle-treated mice (Figure 2.6E). Thus, the increase in the local 1,25(OH)₂D₃ concentration by NLC-D3 may suppress the recruitment of inflammatory PMN cells and reciprocally augment anti-inflammatory CX₃CR1^{high} macrophages.

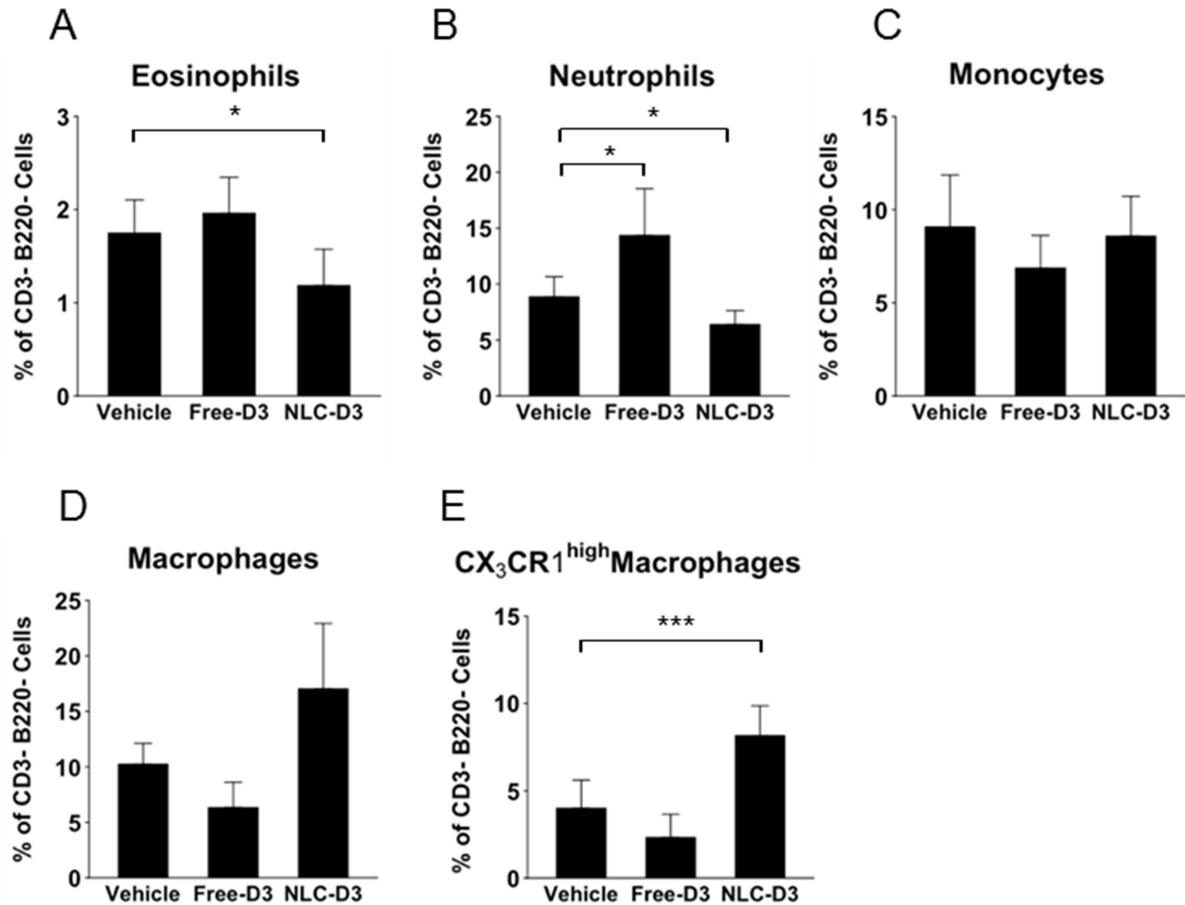


Figure 2.6. Influence of NLC-D3 on the composition of immune cell subsets in the colonic lamina propria. (A–F) Colonic lamina propria cells of the mice treated with vehicle, free-D3 or NLC-D3 were analyzed 7 days after the beginning of DSS administration. Each immune cell subset was characterized based on the surface expression of specific markers. The frequency of eosinophils (A), neutrophils (B), monocytes (C), total macrophages (D), and CX₃CR1^{high} macrophages (E) in the CD3-B220- cell gate are shown. Values are the mean $\pm$ SD (n = 6). *P < 0.05, ***P < 0.001 vs vehicle control. Data are representative of two independent experiments.

2.3 Summary

The active form of vitamin D₃, 1,25(OH)₂D₃ has been found to exert multiple effects on the suppression of progression of inflammatory bowel disease (IBD). Vitamin D₃ has been gathering attention as a therapy for IBD. However, the clinical trials conducted to date revealed that a relatively high dosage of vitamin D₃ was required to see a significant therapeutic effect. Thus, effective formulation and delivery of vitamin D₃ to colonic inflammatory lesions will be required. Herein I established procedures for the efficient incorporation of intact 1,25(OH)₂D₃ into a NLC by employing organic solvents for emulsification at ambient temperature and including anti-oxidative components (α -tocopherol, ascorbic acid) to protect 1,25(OH)₂D₃ from radical species. The NLC-D3 thus obtained showed significant *in vitro* suppression of the expression of proinflammatory cytokines in macrophages. NLC-D3 successfully maintained a high concentration of 1,25(OH)₂D₃ in colonic tissue for at least 12 h. The continuous release of 1,25(OH)₂D₃ from NLC-D3 enabled significant suppression of the progression of DSS colitis using a small dosage of 1,25(OH)₂D₃. NLC-D3 suppressed the infiltration of eosinophils and neutrophils but conversely raised the population of CX₃CR1^{high} anti-inflammatory macrophages. Despite the simple formulation, NLC-D3 provided the desired therapeutic effect in an IBD model by oral administration.

The NLC showed multiple effects on the suppression of symptoms of colitis induced by DSS, namely maintaining crypt structure, reducing the tissue concentration of inflammatory cytokines, suppressing the infiltration of polymorphonuclear leukocytes, and augmenting anti-inflammatory CX₃CR1^{high} macrophages. NLC-D3 may be an alternative intervention for IBD because it acts through a different therapeutic mechanism from the currently available anti-inflammatory and immunosuppressive drugs.

2.4 Experimental section

Materials

L- α Phosphatidylcholine (PC) from soybean, Pluronic F-127 and α -tocopherol were purchased from Sigma-Aldrich. 1,25(OH) $_2$ D $_3$ was purchased from Cayman Chemical Company. L-(+)-Ascorbic acid, phosphotungstic acid and glycerol monostearate (GMS) were purchased from Wako. L-3-Phosphatidyl-L-serine sodium (PS) from bovine was purchased from Olbracht Cedary Research Laboratories. 3,3'-Diocetadecyloxacarbocyanine perchlorate (DiO) was purchased from Takara-Clontech.

Preparation of the NLCs

NLCs containing 1,25(OH) $_2$ D $_3$ were prepared by an oil in water emulsion method. A total of 1.0 mL of a chloroform-toluene (v/v = 1/1; density 1.1 g/cm ^{3}) mixed solution containing GMS (30 mg/mL), PC (4 mg/mL), PS (1 mg/mL), α -tocopherol (2 mg/mL), and 1,25(OH) $_2$ D $_3$ (20 μ g/ml) was prepared. Then this organic solution was added to an aqueous solution (24 mL) containing Pluronic F127 (1.25 mg/mL) and ascorbic acid (0.42 mg/mL). The two-phase solution was mixed by homogenizer (T25 digital Ultra turax, IKA, Germany) at 22,000 rpm for 15 min and sonicated with a probe sonicator (Ultrasonic Disrupter UD 201 (TOMY) equipped with a TP 040 standard probe tip) for 5 min (10 % power, 20 kHz, 50 W). Then the organic solvent was evaporated from the solution by stirring (300 rpm) overnight. Fluorescently labeled NLCs were similarly prepared by mixing with DiO (10 μ g/mL) in the organic phase. PS-free NLCs were prepared by using only PC (5 mg/mL) as a phospholipid component in the organic phase.

Measurement of the NLC size and ζ -potential

The size, polydispersity index, and ζ -potential of the NLCs were measured with a dynamic light scattering spectrophotometer (Zeta sizer Nano series, Malvern Instruments, UK) at 25 °C. To measure the size and polydispersity index, the NLC dispersions were diluted with water to a concentration of 0.25 mg/mL of GMS. For measurement of the ζ -potential, the NLC dispersions were diluted with 10 mM HEPES buffer (pH 7.4) to a concentration of 0.25 mg/mL of GMS.

Transmission electron microscopy

A NLC dispersion was dropped on a 150 mesh-copper grid, then the NLC was stained with 2% phosphotungstic acid. The grid was air dried at room temperature, then observed by using a transmission electron microscopy (TEM) (JEOL JEM-2010, USA) operated at 120 keV.

Determination of the entrapment efficiency of 1,25(OH)₂D₃ in the NLC

The prepared NLC dispersion (5 mL) was ultracentrifuged at 220,000 g for 45 min. After collecting the supernatant, the NLC pellet was dissolved in methanol/chloroform at a ratio of 1:1. The amount of intact 1,25(OH)₂D₃ in the NLC and supernatant (aqueous phase) was quantified by HPLC using an Elite system (Hitachi, Japan) equipped with a Sunfire C18 OBD Prep column (100Å, 5 µm, 10 mm×150 mm). An isocratic mobile phase consisting of 10% of acetonitrile and 90% of methanol was used with a flow rate of 1.0 ml/min. The UV detector was set to 260 nm. A 20 µL aliquot of sample was injected for analysis.

Cell culture

RAW 264.7 macrophages and RAW 264.7 macrophages transfected with secreted alkaline phosphatase (SEAP) as a reporter gene under the transcriptional control of an NF-κB response element (Novusbio) were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 4 mM L-glutamine supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 1 mM sodium pyruvate, and 500 µg/mL G418 (only for the RAW 264.7 macrophages transfected with SEAP). The cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Cellular uptake of NLC

RAW 264.7 macrophages (1×10⁴ cells/well) were seeded in a 96-well glass surface plate with complete DMEM. After 24 h of incubation, the cells were washed twice with DPBS. Complete DMEM containing 0.01% NLC-DiO were added to the well. After 6 h and 24 h of incubation at 37°C, the cells were subjected to a Tali Image-Based Cytometer (Life Technologies) to quantify the ratio of positive cells and to LSM700 confocal laser scanning fluorescence microscopy (Carl Zeiss), respectively.

Cytotoxicity of NLC

RAW 264.7 macrophages (4×10^4 cells/well) were seeded in a 48-well plate with complete DMEM. After 24 h of incubation, the medium was replaced with complete DMEM containing 0.01% NLC or NLC-D3 and incubated for 24 h at 37°C. Cell viability was assessed using the LDH Assay Kit-WST (DOJINDO Laboratories).

NF- κ B-mediated inflammatory response

RAW 264.7 macrophages transfected with SEAP (4×10^4 cells/well) were cultured in a 48-well plate with complete DMEM. After incubation overnight, the medium was replaced with complete DMEM containing 0.01% NLC. After 6 h, LPS (final conc. 20 ng/mL) was added and the plate was incubated for 18 h. After incubation, the supernatants were mixed with an equal volume of alkaline phosphatase substrate (1 mg/mL p-nitrophenylphosphate) and incubated at room temperature for 1.5 h and then 3 N NaOH was added to stop the reaction. The optical density of the solution was measured at 405 nm with a microplate reader (Wallac ARVO.SX 1420 Multilabel counter).

Gene expression of cytokines *in vitro*

RAW 264.7 macrophages (4×10^5 cells/well) were seeded in a 6-well plate with complete DMEM. After incubation overnight, the medium was replaced with complete DMEM containing 0.01% NLC-D3. After 6 h, LPS (final conc. 10 ng/mL) was added and the cells were incubated for 18 h. The total RNA from the cells was prepared using Isoplus reagent (Takara). The samples were reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) and the synthesized cDNA was used as a template in qPCR experiments performed with a LightCycler 1.5 (Roche Diagnostics, Germany) and analyzed with LightCycler Manager software 3.5 (Roche Diagnostics, Germany). The relative expression level was calculated by the $\Delta\Delta C_t$ method using *Gapdh* as a reference gene. All primers were purchased from Takara-Clontech Laboratories (Japan). The sequences of the primer sets are listed in the table below.

Transcript	Forward primer	Reverse primer
<i>Il1b</i>	5'-GCAACTGTTCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
<i>Il6</i>	5'-CCACTTCACAAGTCGGAGGCTTA-3'	5'-CCAGTTTGGTAGCATCCATCATTTC-3'
<i>Tnf</i>	5'-ACCCTCACACTCAGATCATCTTC-3'	5'-TGGTGGTTTGCTACGACGT-3'
<i>Gapdh</i>	5'-CCCAGCAAGGACACTGAGCAAG-3'	5'-GGTCTGGGATGGAAATTGTGAGGG-3'

***In vivo* assessment of vitamin D3 in colon tissue**

Male C57BL/6J mice were purchased from Kyudo, Co., Ltd. (Saga, Japan) and were maintained under standard conditions in the animal facility of Kyushu University. Animal studies were performed with the approval of the Ethics Committee for Animal Experiments and in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan). Mice received vehicle (0.0262% EtOH, free-D3 (0.262 µg/ml of 1,25(OH)₂D₃), or NLC-D3 (0.262 µg/ml of 1,25(OH)₂D₃; 625 µg/ml of NLCs) by oral gavage at a dose of 10 ml/kg b.w. once. After 6 to 12 h of oral gavage administration of free-D3 or NLC-D3, the mice were sacrificed and the colon tissue was cut from the mice. The colon tissue was washed in PBS to remove blood and feces and then samples were minced with scissors to disrupt connective tissue prior to transfer into tubes. 1,25(OH)₂D₃ was extracted following Wagner's method [36]. Briefly, tissue samples were reconstituted by first immersing them into 1 mL distilled water and then adding 3.75 mL of a mixture of methanol/dichloromethane (2:1). The reconstituted tissue was homogenized at high speed for 5 minutes using a homogenizer (T10 digital Ultra turax, IKA, Germany). Next, dichloromethane was added to the mixture (1.25 mL), followed by distilled water (1.25 mL). All samples were vortexed thoroughly after the addition of each reagent. After centrifugation (1100 g, 20 minutes), the lower lipid-containing layer was collected and the remaining protein residue was re-extracted with methylene chloride (1.25 mL). The collected dichloromethane extracts were freeze dried. The levels of 1,25(OH)₂D₃ in the tissue extracts were measured using the 1,25(OH)₂D₃ EIA kit following the manufacturer's protocol (Immunodiagnosics Systems, Scottsdale, AZ).

DSS-induced colitis model study

C57BL/6J mice were purchased from CLEA Japan, Inc (Tokyo, Japan) and were maintained under SPF conditions in the animal facility of Keio University. All studies were approved by the Animal Care Committee of Keio University. Colitis was induced by oral administration of dextran sodium sulfate (DSS; 36,000–50,000 MW; MP Biomedicals, LLC, Santa Ana, CA, USA) in drinking water until Day 6. Then the mice were given standard drinking water without DSS, and analyzed on Day 7. During the experiment, mice received vehicle (0.0262% EtOH), free-D3 (0.262 µg/ml of 1,25(OH)₂D₃), or NLC-D3 (0.262 µg/ml of 1,25(OH)₂D₃; 625 µg/ml of NLCs) by oral gavage at a dose of 10 ml/kg b.w. once a day from Day 0. Body weight and clinical score were monitored daily. Clinical score was

determined according to previously reported criteria [37] on the basis of the stool consistency (0, normal; 1, soft but still formed; 2, very soft; 3, diarrhea) and hematochezia (0, negative occult blood; 1, positive occult blood; 2, blood traces in stool visible; 3, rectal bleeding). Fecal occult blood was examined using ColoScreen-ES (Helena Laboratories, Beaumont, TX, USA).

Histology

Colonic tissue samples were fixed in 10% formalin neutral buffer solution (Mildform 10N, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) overnight. After fixation, the samples were embedded in paraffin and then cut into 3 μm sections. The sections were deparaffinized, rehydrated and stained with hematoxylin (Agilent Technologies, Inc., Santa Clara, CA, USA) and eosin (Wako) for hematoxylin and eosin staining or with Alcian blue solution (Wako) followed by nuclear fast red (Vector Laboratories, Inc., Burlingame, CA, USA) for Alcian blue staining and then mounted with Mount-Quick (Daido Sangyo Co., Ltd., Tokyo, Japan). The specimens were subjected to histological examination. The histological score was determined by the following criteria: crypt architecture, inflammatory cell infiltration and goblet cell depletion.

Preparation of colonic lamina propria cells

Colonic lamina propria cells were prepared as described previously [38] with some modifications. The colons were isolated, opened longitudinally, washed with HBSS (Nacalai Tesque, Inc., Kyoto, Japan), cut into four segments and stirred in a glass container on a magnetic stirrer in HBSS containing 1 mM dithiothreitol, 20 mM EDTA and 12.5 mM HEPES (Nacalai Tesque) at 37°C for 20 min. After vortexing, the colonic tissues were centrifuged at 70 $\times$ g for 30 sec. After the supernatant was discarded, the precipitated tissue was suspended in HBSS containing 20 mM EDTA and 12.5 mM HEPES and stirred in a glass container using a magnetic stirrer at 37°C for 20 min. The tissue samples were then minced and dissociated with RPMI1640 containing 0.2 U/ml Liberase TM (Roche Diagnostics, Mannheim, Germany), 0.125 mg/mL DNase I (Merck, Darmstadt, Germany), 2% FCS (MP Biomedicals), 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (Nacalai Tesque) and 25 mM HEPES at 37°C for 30 min to obtain single-cell suspensions. After filtering with 70- μm mesh filters, the single-cell suspensions were washed with HBSS and subjected to flow cytometry analysis.

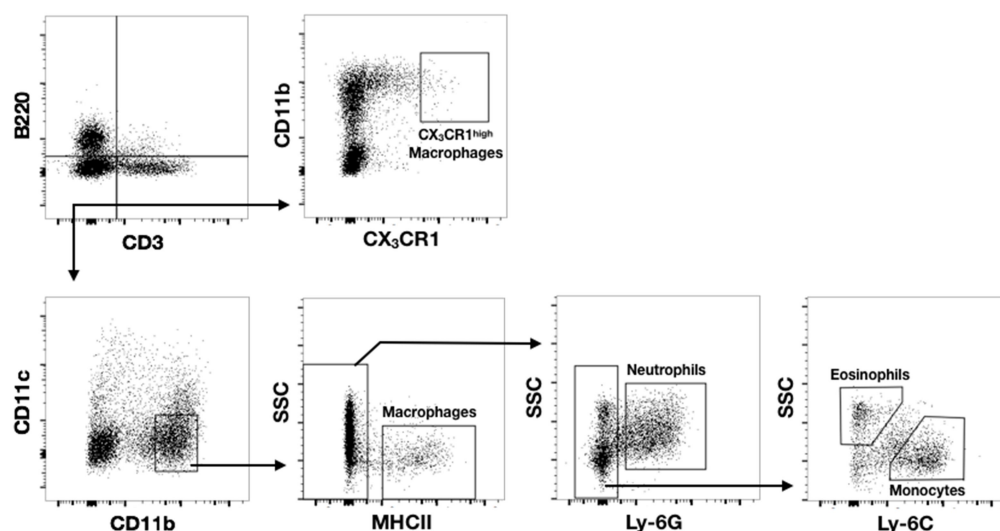
Flow cytometry of colonic lamina propria cells

Colonic lamina propria cells were incubated with anti-mouse CD16/CD32 antibody (93; BioLegend, Inc., San Diego, CA, USA) to block Fc receptors and then stained using antibodies conjugated with fluorescein isothiocyanate, phycoerythrin (PE), PE-Cy7, allophycocyanin (APC), APC-Cy7, redFluor 710, eFluor 450, Brilliant Violet 510 or Brilliant Ultraviolet 395. Antibodies against CD11b (M1/70), B220/CD45R (RA3-6B2), CD45 (30-F11) and Ly6C (Al-21) were obtained from BD Biosciences (San Jose, CA, USA), anti-CX₃CR1 (SA011F11) antibody was from BioLegend, MHC Class II (I-A/I-E) antibody was from Thermo Fisher Scientific (Waltham, MA, USA), and antibodies against CD11c (N418), CD3 (17A2) and Ly6G (1A8) were obtained from Tonbo Biosciences (San Diego, CA, USA). 7-AAD (BioLegend) was added to the cell suspension to label dead cells. The cells were analyzed by a LSR II Flow Cytometer (BD Biosciences).

Surface marker used to identify immune cells in Fig. 2.6.

Surface marker	Type of cell
CD3	T cells
CD11b	leukocytes, monocytes, neutrophils, natural killer cells, granulocytes and macrophages
CX ₃ CR1 ^{high}	Macrophages
CD11c	Monocytes, macrophages, natural killer cells
MHC II	Macrophages
Ly-6G	Neutrophils
Ly-6C	Monocyte

Procedure of identification of immune cell subsets in the colonic lamina propria.



qPCR of colonic lamina propria cells

The total RNA from colonic tissues was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The samples were reverse transcribed using an iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the synthesized cDNA was used as a template in qPCR experiments with SsoAdvanced Universal SYBR Green Supermix and analyzed with a CFX96 Real-time system equipped with CFX Manager software (Bio-Rad Laboratories). The relative expression level was calculated by the $\Delta\Delta C_t$ method using *Tbp* as a reference gene unless otherwise stated. All primers were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The sequences of the primer sets are listed in the table below.

Transcript	Forward primer	Reverse primer
<i>Il1b</i>	5'-CGACAAAATACCTGTGGCCT-3'	5'-TTCTTTGGGTATTGCTTGGG-3'
<i>Il6</i>	5'-GTTCTCTGGGAAATCGTGGA-3'	5'-TTCTGCAAGTGCATCATCGT-3'
<i>Tnf</i>	5'-AGTTCTATGGCCCAGACCCT-3'	5'-CTCCTCCACTTGGTGTTTG-3'
<i>Tbp</i>	5'-CCCAGCAAGGACACTGAGCAAG-3'	5'-GGTCTGGGATGGAAATTGTGAGGG-3'

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CHAPTER 3

Regulation of inflammatory response of macrophages and induction of regulatory T cells by using retinoic acid-loaded nanostructured lipid carrier

3.1 Introduction

All-trans retinoic acid (ATRA), which is the active metabolite of vitamin A, is an agonist of nuclear receptors, retinoic acid receptors (RARs) [1-6]. ATRA induces macrophages and dendritic cells to express anti-inflammatory cytokines via RARs, which work on these cells to shift their phenotype to be anti-inflammatory and tolerogenic, respectively [1-6]. These anti-inflammatory macrophages and tolerogenic dendritic cells induce the naïve T cells to regulatory T (Treg) cells, which suppress excessive inflammatory responses [7] and induce immunotolerance toward antigens [1-6].

Because of these immunomodulatory functions of ATRA, therapeutic effects of ATRA have been examined for autoimmune diseases such as inflammatory bowel disease (IBD) [4-7], rheumatoid arthritis [8], asthma [10, 11], and systemic lupus erythematosus [12, 13]. However, chemical instability [14], poor water solubility [15], and short half-life in blood [16], of ATRA complicates its dosage formulation. To overcome these limitations, I tried to incorporate ATRA in nanostructured lipid carriers (NLC) that will improve stability and the therapeutic efficacy of ATRA. NLC is formulated from combination of a solid lipid with a liquid lipid to raise drug entrapment efficiency [17]. NLC has been originally developed for oral administration because of its stable nature in gastric fluid [18]. Thus, NLC will be suitable carrier to deliver ATRA to inflammatory lesions in gastrointestinal tract for IBD therapy.

Here I established preparation procedure of NLC containing ATRA (NLC-RA) to minimize the degradation of ATRA during preparation. As schematically shown in Figure 3.1, I evaluated the ability of NLC-RA to induce anti-inflammatory response in macrophages as well as differentiation of naïve T cells to Treg in vitro.

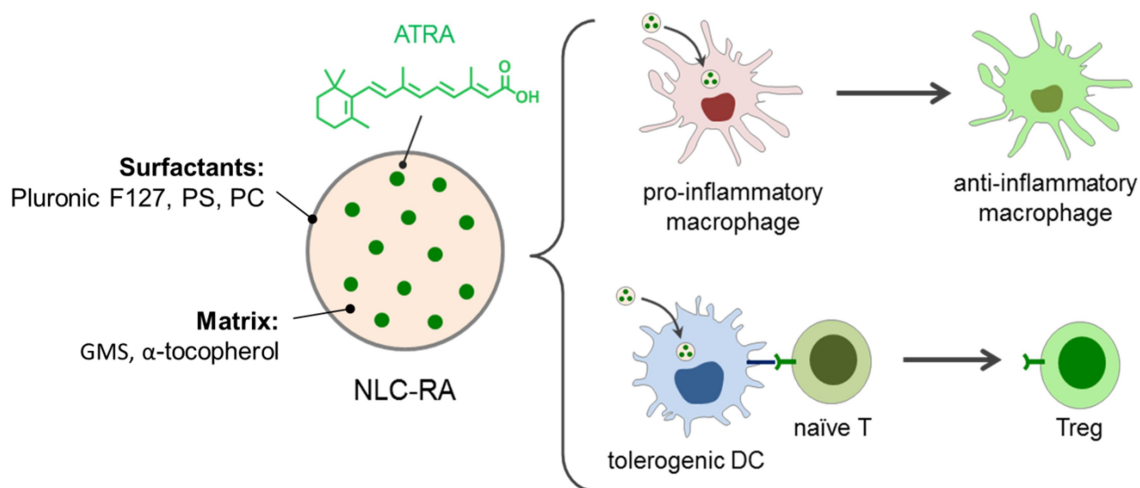


Figure 3.1. Modulation of macrophages and T cells by ATRA-containing NLC (NLC-RA) for inflammatory resolution.

3.2 Results and discussion

3.2.1 Preparation and characterization of NLC

The conventional preparation procedures of NLC use high temperature to melt solid lipid component to prepare pre-emulsion, then the size of the pre-emulsion is reduced by ultrasonication or high-pressure homogenizer [19, 20]. Because ATRA is sensitive to high temperatures [14], the conventional preparation procedures of NLC are not suited. To avoid high temperatures, I prepared NLC containing ATRA (NLC-RA) by using organic solvent to dissolve lipids and ATRA to prepare the pre-emulsion at ambient temperature. The formulation of NLC consists of a core matrix of glycerol monostearate and α -tocopherol as a solid and a liquid lipid, respectively. α -Tocopherol is antioxidant, which is expected to suppress inflammation by neutralizing reactive oxygen species (ROS) [21, 22]. The anti-oxidative function of α -tocopherol may contribute to protect ATRA from radical species which generate during preparation of NLC. As stabilizers of NLC, I used combination of a neutral polymer (Pluronic F127) and phospholipids. All the components of NLC I selected are approved materials for clinical application.

I tried to establish preparation conditions to obtain NLC with small size (~ 100 nm) with high entrapment efficiency of ATRA. Nanoparticles with size of about 100 nm is suitable to target inflammatory regions in gastrointestinal tract [20]. Table 3.1 summarizes

characteristics of NLC-RA prepared by various conditions. Homogenization alone or ultrasonication alone did not produce NLC-RA with small particle size (run 1-4). Moreover, the ultrasonication alone reduced the entrapment efficacy of ATRA (run 3, 4). In contrast, combination of the homogenization and the ultrasonication (run 5) produced NLC-RA with a mean size of about 130 nm, narrow size distribution, and high entrapment efficacy of ATRA. Morphology of NLC-RA prepared by the condition of run 5 was found to be spherical shape with sizes of approximately 50-200 nm (Figure 3.2), which is matched with the result of dynamic light scattering. I concluded that the condition of run 5 is optimum for the preparation of NLC-RA. By using this condition, empty NLC (NLC) and fluorophore containing NLC (NLC-DiO) were prepared (Table 3.2). Size, polydispersity index and ζ -potential of each NLC are almost constant irrespective of the content.

Table 3.1. Summary of result of NLC preparation. Data are expressed as mean $\pm$ SD ($n = 3$).

run	condition		size (nm)	polydispersity index	ATRA in NLC (μ g)	ATRA in aq. phase (μ g)	entrapment efficacy (%)
	homogenization	ultrasonication					
1	15 min	-	427 $\pm$ 20	0.66 $\pm$ 0.04	365.6 $\pm$ 24.6	128.0 $\pm$ 0.5	98.7 $\pm$ 4.8
2 ^a	-	10%, 5 min	-	-	-	-	-
3	-	10%, 15 min	429 $\pm$ 22	0.64 $\pm$ 0.02	213.0 $\pm$ 10.8	56.8 $\pm$ 1.0	53.9 $\pm$ 2.1
4	-	20%, 10 min	179 $\pm$ 9	0.44 $\pm$ 0.02	204.8 $\pm$ 6.1	77.4 $\pm$ 0.7	56.4 $\pm$ 1.3
5	15 min	10%, 5 min	130 $\pm$ 9	0.29 $\pm$ 0.02	312.3 $\pm$ 5.5	49.1 $\pm$ 4.7	72.3 $\pm$ 1.4

Table 3.2. Summary of characteristics of NLCs in 10 mM HEPES (pH 7.4). Data are expressed as mean $\pm$ SD ($n = 3$)

Particle	content	Size (nm)	Polydispersity index	ζ -potential (mV)
NLC	-	108 $\pm$ 6	0.23 $\pm$ 0.02	-17.6 $\pm$ 0.9
NLC-RA	ATRA	123 $\pm$ 9	0.29 $\pm$ 0.02	-18.2 $\pm$ 2.6
NLC-DiO	DiO	107 $\pm$ 7	0.24 $\pm$ 0.00	-16.7 $\pm$ 0.9

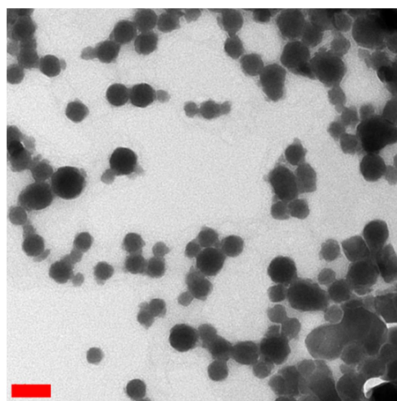


Figure 3.2. TEM image of NLC-RA. Scale bar: 100 nm.

3.2.2 Cellular uptake and cytotoxicity of NLC

I evaluated cellular uptake of NLC-DiO by RAW 264.7 macrophages. The uptake of NLC-DiO was assessed by confocal laser scanning microscopy at 24 h after incubation. As shown in Figure 3.3, the strong green fluorescence of DiO was observed in the macrophages. I examined the cytotoxicity of NLC and NLC-RA against macrophages after 24 h incubation. Despite the high cellular uptake of NLC, I observed no cytotoxicity of either NLC or NLC-RA below the concentration of 0.5 mg/mL (Figure 3.4), showing cytocompatible nature of NLC.

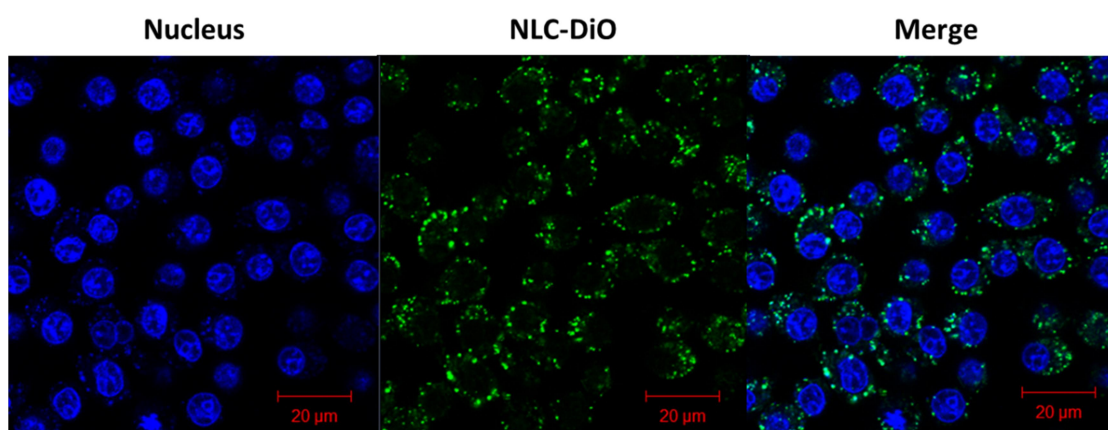


Figure 3.3. Cellular uptake of NLC-DiO by RAW 264.7 macrophages. Macrophages were treated with 0.1 mg/mL NLC-DiO and incubated for 24 h at 37°C. The images were taken with a confocal laser scanning fluorescence microscopy. Blue and green colors represent nucleus and NLC-DiO, respectively.

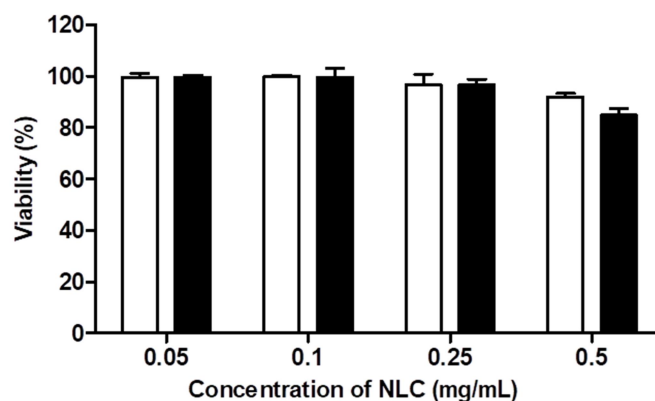


Figure 3.4. Viability of RAW 264.7 macrophages treated with NLC (white bar) and NLC-RA (filled bar) for 24 h, respectively. Data in each bar are the mean $\pm$ SD ($n = 3$).

3.2.3 NLC-RA inhibits LPS-induced NF- κ B signaling

I investigated the effect of NLC-RA on inhibition of NF- κ B signaling triggered by LPS stimulation in macrophages. NF- κ B is one of the key transcription factors in activation of macrophages during inflammation process [23]. Here I utilized RAW 264.7 macrophages stably transfected with secreted alkaline phosphatase (SEAP) reporter gene under the transcriptional control of an NF- κ B response element. As shown in Figure 3.5, both NLC and NLC-RA showed suppression of NF- κ B signaling, but NLC-RA has somewhat superior effect to NLC. The suppressive effect of empty NLC will be due to the anti-inflammatory effect of α -tocopherol used as a component of NLC.

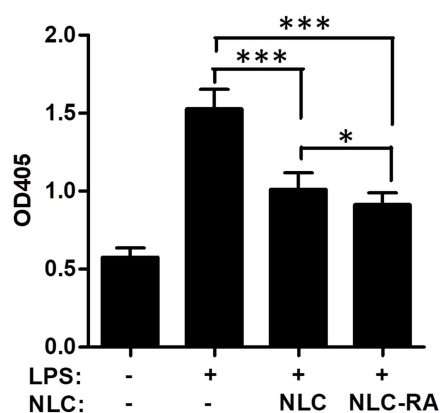


Figure 3.5. Suppression of NF- κ B signaling in LPS-stimulated RAW 264.7 macrophages by NLCs. Macrophages were treated with NLC and NLC-RA (0.1 mg/mL containing 3.36 μ M ATRA). Data are the mean $\pm$ S.D ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$

3.2.4 NLC-RA alters cytokines gene expression in macrophages

I assessed the effect of NLC-RA on the expression of pro- and anti-inflammatory cytokines from the LPS-stimulated RAW 264.7 macrophages. As shown in Figure 3.6, NLC-RA significantly suppressed the expression of mRNA of pro-inflammatory cytokines (IL-6, IL-1 β , TNF- α), while strongly enhanced that of anti-inflammatory cytokines (IL-10, TGF- β). Similar results were obtained when the expression of the cytokines was detected as proteins; suppression of IL-6 and enhancement of IL-10 (Figure 3.7). IL-10 and TGF- β which were enhanced by NLC-RA are the most important anti-inflammatory cytokines in the resolution of inflammation and TGF- β is required to differentiate naïve T cells to Treg [24]. Thus, NLC-RA will have effect to induce Treg as well as to suppress the inflammation.

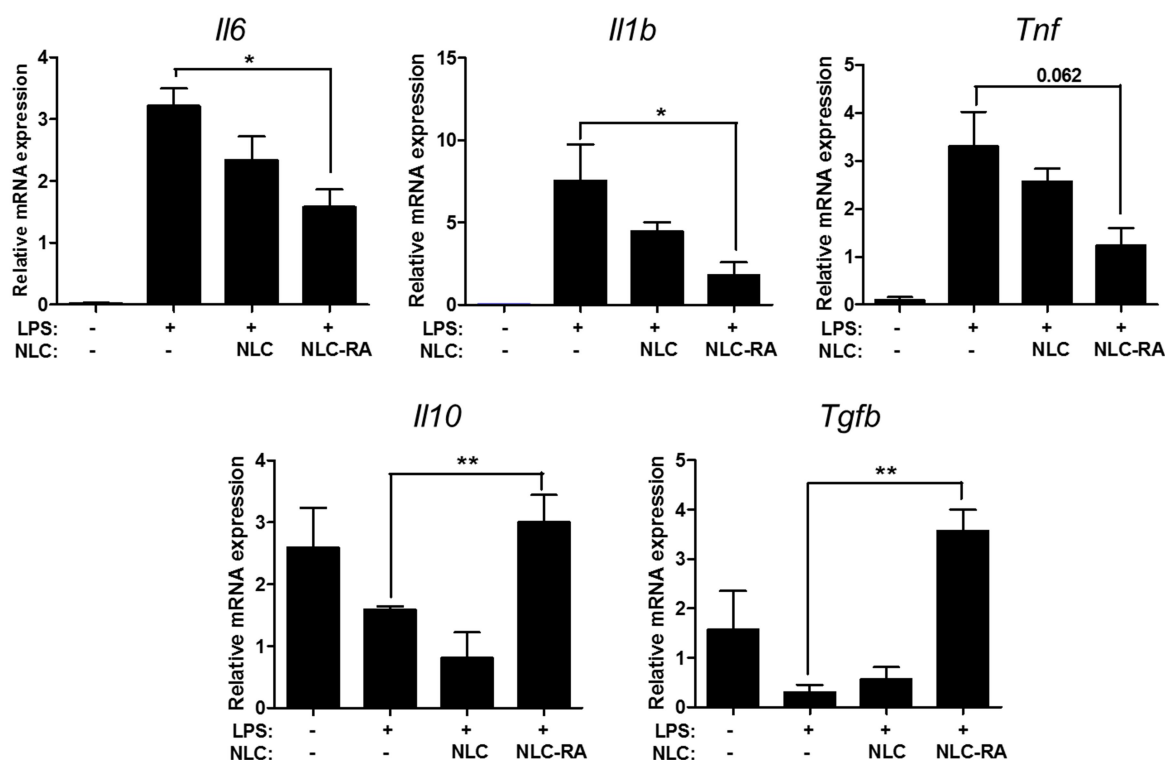


Figure 3.6. Modulation of gene expression of pro- and anti-inflammatory cytokines in RAW 264.7 macrophages by NLCs. Macrophages were treated with NLC and NLC-RA (0.1 mg/mL containing 3.36 μ M ATRA) and gene expression was evaluated by qRT-PCR. Data are the mean $\pm$ S.D (n = 3). *, $p < 0.05$; **, $p < 0.01$.

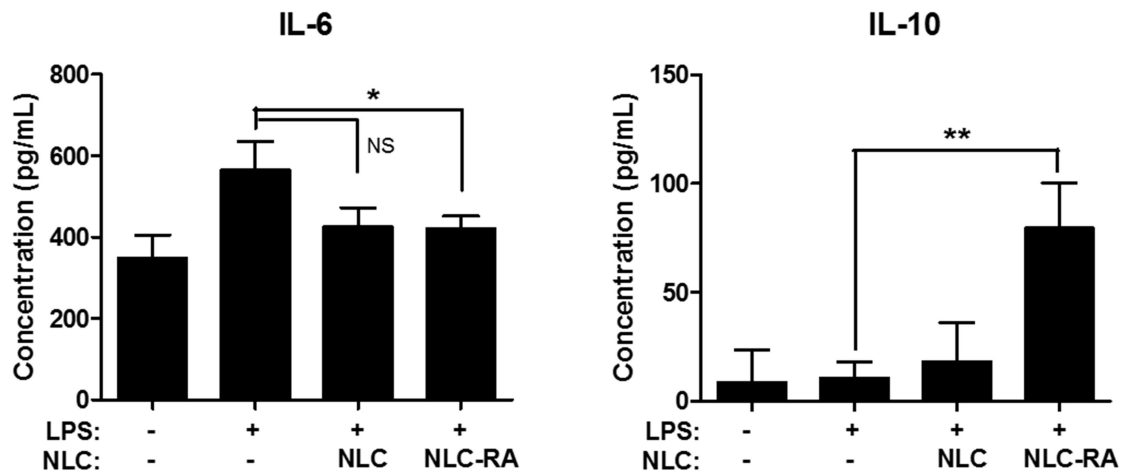


Figure 3.7. Modulation of cytokine production of IL-6 and IL-10 in RAW 264.7 macrophages by NLCs. Macrophages were treated with NLC and NLC-RA (0.25 mg/mL containing 8.4 μ M ATRA). Data are mean $\pm$ S.D (n = 3). *, $p < 0.05$; **, $p < 0.01$.

3.2.5 Induction of Treg by NLC-RA

I examined effect of NLC-RA in the differentiation of naïve T cells to Treg by using the co-culture system of naïve T cells and dendritic cells both of which were isolated from mice spleen. As shown in Figure 3.8A, NLC-RA could induce Treg and its induction efficacy of Treg was comparable to that of free ATRA. NLC-RA did not induce cytotoxicity to T cells and dendritic cells (Figure 3.8B). Thus, incorporation of ATRA into NLC did not compromise the induction efficacy of Treg, indicating that NLC matrix could be degraded in the dendritic cells to release ATRA.

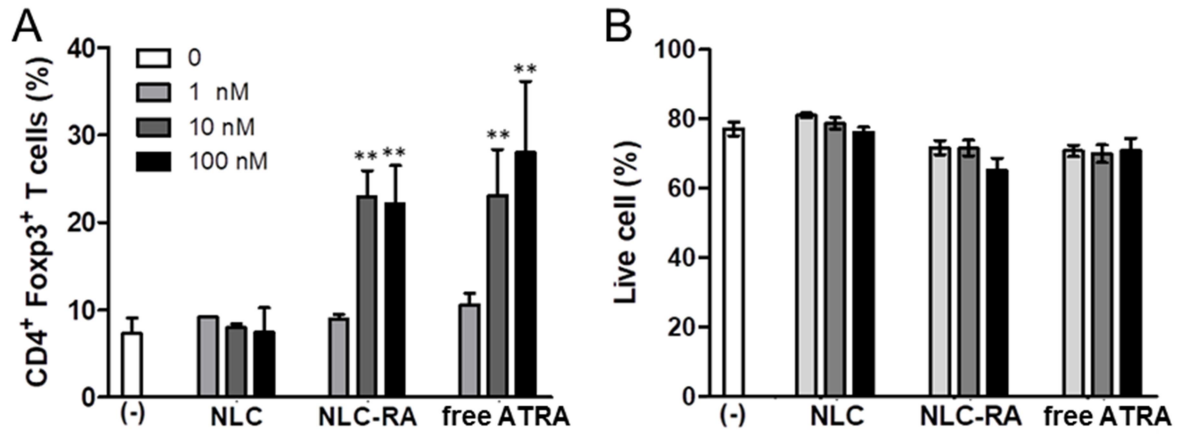


Figure 3.8. Effect of NLCs on promotion of differentiation to CD4⁺Foxp3⁺Treg (A) and cell viability (B) in the co-culture of dendritic cells and CD4⁺CD25⁻ T cells. Cells were treated with NLC, NLC-RA and free ATRA (0.02-2 μ g/ml NLC containing 1-100 nM ATRA). Data are mean $\pm$ S.D (n = 3). **, $p < 0.01$. The reference in t-test is control (-).

3.3 Summary

Immunomodulatory function of all-trans retinoic acid (ATRA) has been gathering much attention for the therapy of autoimmune diseases. ATRA is a chemically unstable molecule which requires proper formulation for targeted delivery. Here I established the preparation procedures of ATRA-containing NLC (NLC-RA) which minimizes the degradation of ATRA during the preparation process. The size of NLC-RA was tuned to be ~100 nm, which is known to be suitable size for oral delivery to target inflammatory lesions of gastrointestinal tract. NLC-RA prepared by the procedure had cytocompatibility to macrophages even though its efficient cellular uptake. NLC-RA reduced the expression of pro-inflammatory cytokines while enhanced that of anti-inflammatory cytokines from the LPS-stimulated macrophages via suppressing NF- κ B signaling as well as via enhancing the production of anti-inflammatory cytokines. Moreover, NLC-RA enhanced differentiation of naïve T cells to regulatory T cells in the co-culture system with dendritic cells. These results suggest that NLC-RA is a promising alternative therapy for the autoimmune diseases especially intestinal bowel disease.

3.4 Experimental section

Materials

L- α Phosphatidylcholine (PC) from soybean, Pluronic F-127 and α -tocopherol were purchased from Sigma-Aldrich. All-trans retinoic acid (ATRA) was purchased from TCI. L-(+)-Ascorbic acid, phosphotungstic acid and glycerol monostearate (GMS) were purchased from Wako. L-3-Phosphatidyl-L-serine sodium (PS) from bovine was purchased from Olbracht cedary research laboratories. 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from Takara-Clontech.

Preparation of NLC-RA

NLCs containing ATRA were prepared by an oil in water emulsion method. A total of 1.0 mL of a chloroform-toluene ($v/v = 1/1$; density 1.1 g/cm^3) mixed solution containing GMS (30 mg/mL), PC (4 mg/mL), PS (1 mg/mL), α -tocopherol (2 mg/mL), and ATRA (500 $\mu\text{g/ml}$) was prepared. Then this organic solution was added to an aqueous solution (24 mL) containing Pluronic F127 (1.25 mg/mL) and ascorbic acid (0.42 mg/mL). The two-phase solution was mixed by homogenizer (T25 digital Ultra turax, IKA, Germany) at 22,000 rpm for 15 min and sonicated with a probe sonicator (Ultrasonic Disrupter UD 201 (TOMY) equipped with a TP 040 standard probe tip) for 5 min (10 % power, 20 kHz, 50 W). Then the organic solvent was evaporated from the solution by stirring (300 rpm) overnight. Fluorescently labeled NLCs were similarly prepared by mixing with DiO (10 $\mu\text{g/mL}$) in the organic phase.

Size and ζ -potentials measurements of NLC

The size, polydispersity index, and ζ -potential of the NLCs were measured with a dynamic light scattering spectrophotometer (Zeta sizer Nano series, Malvern Instruments, UK) at 25 °C. To measure the size and polydispersity index, the NLC dispersions were diluted with water to a concentration of 0.25 mg/mL of GMS. For measurement of the ζ -potential, the NLC dispersions were diluted with 10 mM HEPES buffer (pH 7.4) to a concentration of 0.25 mg/mL of GMS.

Transmission electron microscopy

A NLC dispersion was dropped on a 150 mesh-copper grid, then the NLC was stained with 2% phosphotungstic acid. The grid was air dried at room temperature, then observed by

using a transmission electron microscopy (TEM) (JEOL JEM-2010, USA) operated at 120 keV.

Quantification of intact ATRA in NLC

The prepared NLC dispersion (1 mL) was ultracentrifugation at 220,000 g for 45 min. After collecting the supernatant, the NLC pellet was dissolved in methanol/chloroform at a ratio of 1:1. The amount of intact ATRA in NLC and supernatant (aqueous phase) was quantified by Jasco V-670 spectrophotometer (UK).

Cell culture

RAW 264.7 macrophages transfected with secreted alkaline phosphatase (SEAP) as a reporter gene under the transcriptional control of an NF- κ B response element (Novusbio) were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 4 mM L-glutamine supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/ mL amphotericin B, 1 mM sodium pyruvate, and 500 μ g/mL G418. The cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Cellular uptake of NLC

RAW 264.7 macrophages transfected with SEAP (1×10^4 cells/well) were seeded in a 96-well glass surface plate with complete DMEM. After 24 h of incubation, the cells were washed twice with DPBS. Complete DMEM containing 0.1 mg/mL NLC-DiO was added to the well. After 24 h of incubation at 37°C, the cells were observed for the uptake of NLC-DiO by LSM700 confocal laser scanning fluorescence microscopy (Carl Zeiss).

Toxicity of NLC

RAW 264.7 macrophages transfected with SEAP (4×10^4 cells/well) were seeded in a 48-well plate with complete DMEM. After 24 h of incubation, the medium was replaced with complete DMEM containing NLC or NLC-RA (1.68-16.8 μ M of ATRA; 0.05-0.5 mg/mL of NLC) and incubated for 24 h at 37°C. Cell viability was assessed using the LDH Assay Kit-WST (DOJINDO Laboratories).

Suppression NF- κ B-mediated inflammatory response

RAW 264.7 macrophages transfected with SEAP (4×10^4 cells/well) were cultured in a 48-well plate with complete DMEM. After incubation overnight, the medium was replaced with complete DMEM containing NLC-RA (3.36 μ M of ATRA; 0.1 mg/mL of NLC). After 6 h, LPS (final conc. 20 ng/mL) was added and the plate was incubated for 18 h. After incubation, the supernatants were mixed with an equal volume of alkaline phosphatase substrate (1 mg/mL p-nitrophenylphosphate) and incubated at room temperature for 1.5 h and then 3 N NaOH was added to stop the reaction. The optical density of the solution was measured at 405 nm with a microplate reader (Wallac ARVO.SX 1420 Multilabel counter).

Gene expression of cytokines

RAW 264.7 macrophages transfected with SEAP (4×10^5 cells/well) were seeded in a 6-well plate with complete DMEM. After incubation overnight, the medium was replaced with complete DMEM containing NLC-RA (3.36 μ M of ATRA; 0.1 mg/mL of NLC). After 6 h, LPS (final conc. 10 ng/mL) was added and the cells were incubated for 18 h. The total RNA from the cells was prepared using Isoplus reagent (Takara). The samples were reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) and the synthesized cDNA was used as a template in qPCR experiments performed with a LightCycler 1.5 (Roche Diagnostics, Germany) and analyzed with LightCycler Manager software 3.5 (Roche Diagnostics, Germany). The relative expression level was calculated by the $\Delta\Delta$ Ct method using *Gapdh* as a reference gene. All primers were purchased from Takara-Clontech Laboratories (Japan). The sequences of the primer sets are listed in the table below.

Transcript	Forward primer	Reverse primer
<i>Il1b</i>	5'-GCAACTGTTCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
<i>Il6</i>	5'-CCACTTCACAAGTCGGAGGCTTA-3'	5'-CCAGTTTGGTAGCATCCATCATTTC-3'
<i>Tnf</i>	5'-ACCCTCACACTCAGATCATCTTC-3'	5'-TGGTGGTTTGCTACGACGT-3'
<i>Gapdh</i>	5'-CCCAGCAAGGACACTGAGCAAG-3'	5'-GGTCTGGGATGGAATTGTGAGGG-3'

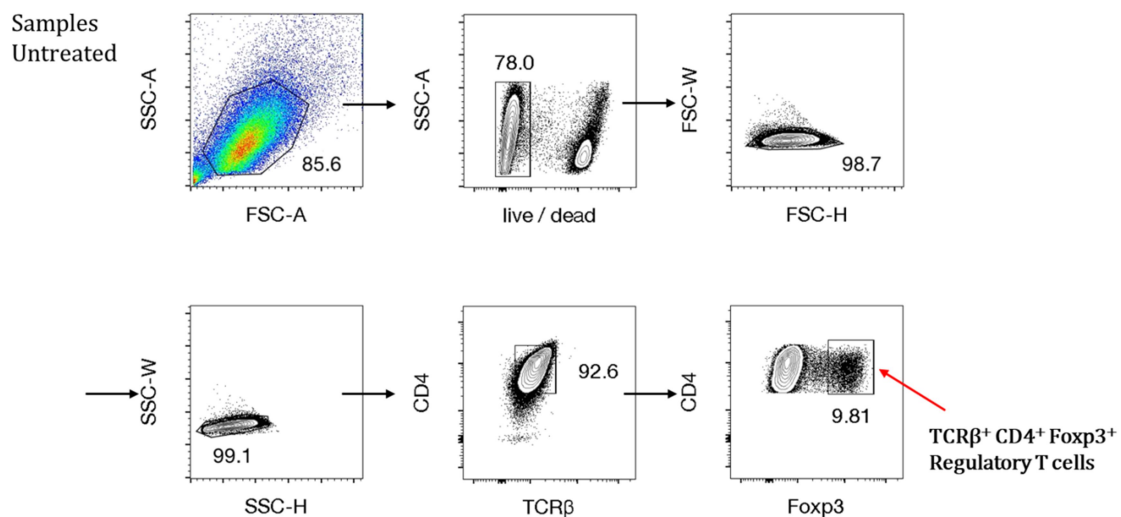
Production of cytokines

RAW 264.7 macrophages transfected with SEAP (1×10^4 cells/well) were seeded in a 48-well plate with complete DMEM. After 24 h of incubation, the medium was replaced with complete DMEM containing NLC or NLC-RA (8.4 μ M of ATRA; 0.25 mg/mL of NLC).

After 24 h, LPS (final conc. 10 ng/mL) was added and the cells were incubated for 24 h. The cell culture supernatant was collected then cytokines were quantified by ELISA kit following the manufacturer's protocol (R&D systems).

Induction of regulatory T cell differentiation

Dendritic cells (DC) and naïve T cells were isolated from C57BL mice spleen cells by magnetic separation (Miltenyi Biotec). DC and naïve T cells were co-cultured in 96-well U-bottom plates (BD Falcon). DC and naïve T cell were seeded at 5×10^3 cells/well and 1×10^5 cells/well respectively. Cultures were maintained in complete RPMI containing anti-CD3 antibody (20 μ g/ml), TGF- β (0.4 ng/ml), and IL-2 (40 ng/ml). NLC-RA or free ATRA (final conc. 1-100 nM of ATRA; 0.02-2 μ g/ml of NLC) was added to complete medium then cells were harvested after 3 days incubation for flow cytometric analysis. Co-culture cells were stained DC and T cell surface using antibodies conjugated with BV510-CD45 (BioLegend) and eF450-MHC II (eBiosciences), FITC-TCR β (BD Biosciences) and PE-CD4 (eBiosciences), respectively. eF660-Foxp3 (eBiosciences) was added to the cell suspension to stain intra cells. FVS-780 (BioLegend) was added to the cell suspension to label dead cells. The cells were analyzed by a LSR II Flow Cytometer (BD Biosciences). Procedure of identification of regulatory T cells in co-culture system is shown below,



3.5 References

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CHAPTER 4

Preparation of complexes between ovalbumin nanoparticles and retinoic acid for efficient induction of tolerogenic dendritic cells

4.1 Introduction

Recently, patients with allergy and autoimmune diseases have been rapidly increasing especially in the developed countries [1-3]. To treat these diseases, immunosuppressing drugs such as steroids and cytokine inhibitors have been utilized. However, these conventional drugs raise the susceptibility for opportunistic infection. Thus, antigen-specific immunotolerance therapy that induces specific tolerance toward allergens and auto-antigens has been gathering attention [4-6]. Tolerogenic dendritic cells (tDCs) play crucial role in the immunotolerance therapy. tDCs show semimatured phenotype, i.e., presenting antigen peptides via major histocompatibility complex (MHC) class II while keeping low level of expression of costimulatory receptors CD80/86 [7, 8]. The semimature phenotype of tDCs induce anergic response to auto-reactive helper T cells as well as differentiate naïve T cells to regulatory T (Treg) cells to maintain tolerance against auto-antigens [7, 8]. Administration of tDCs generated ex vivo has been recognized as a promising therapy of autoimmune diseases and allergy [8].

In vaccination approach of the antigen-specific immunotolerance therapy, antigen peptides are administered to present the peptides on tDCs to induce tolerance. This approach has been applied to the therapy of allergy. However, usually this approach requires relatively long time to obtain therapeutic outcome because dosage of antigen peptides should be small to avoid anaphylactic response [9,10]. Co-administration of inducing molecules of tDCs with antigen peptides will improve therapeutic efficacy of vaccination approach of the immunotolerance therapy. Several molecules such as anti-inflammatory cytokines (IL-10, TGF- β) and small molecules including immunosuppressing drugs and all-trans retinoic acid (ATRA) have been reported to induce tDCs [11-13]. Among these molecules, small molecules will be suitable to prepare formulation for co-administration with antigen proteins. Kishimoto's group reported that biodegradable poly(lactide-co-glycolide) nanoparticles containing antigens and immunosuppressing drug (rapamycin) effectively induce antigen-specific immunotolerance [14,15].

Here we proposed a simple formulation of protein nanoparticles complexed with ATRA (Fig. 1). By using antigen proteins as matrix of nanoparticles, we do not need matrix polymers for the formulation of nanoparticles, which can simplify the formulation procedure. ATRA is an agonist of nuclear receptors and works on DCs as well as naïve T cells to induce semimature DCs and Treg, respectively. We selected ovalbumin (OVA) as a model antigen protein because OVA has been widely used in immunological research. We established preparation procedure of OVA nanoparticles complexed with ATRA and examined their efficacy to induce semimature DC.

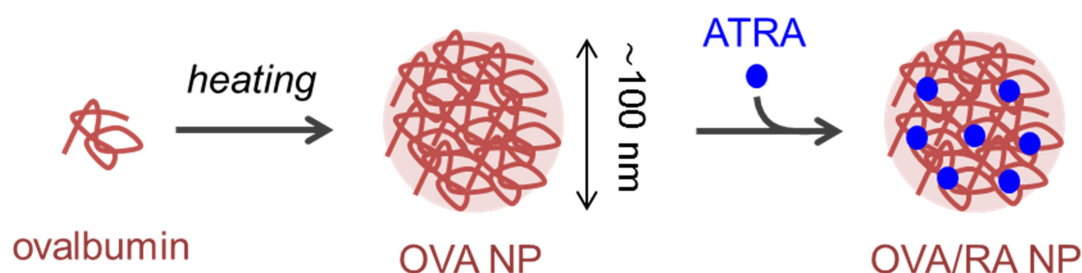


Figure 4.1 Preparation of OVA/RA NP.

4.2 Results and discussion

4.2.1 Preparation and characterization of OVA NPs

To prepare nanoparticles from OVA, we selected heat-induced aggregation method [16, 18, 19]. Because ATRA is unstable to heat, we employed two-step procedure to prepare complex between OVA NPs and ATRA, i.e., preparing OVA NPs then complexing them with ATRA. In the heat-induced aggregation method, size of resulting protein aggregates depends on heating temperature and solution conditions such as a protein concentration, pH and ionic strength [18, 19]. I aimed to adjust the size of OVA NPs around 100 nm because it is ideal size for uptake by dendritic cells [20, 21]. To achieve the aimed size, I screened preparation conditions based on the literature [16] and determined the optimum condition as follows: 10 mg/mL OVA in 50 mM NaCl (pH 7.0), and heating at 85°C for 5 min. Characteristics of the obtained OVA NPs are summarized in Table 4.1 and Figure 4.2. Native OVA became to be OVA NPs with average size of ca. 100 nm. The conformational change of

OVA unit in OVA NPs were confirmed by FT-IR spectra (Figure 4.3). Amide I and amide II bands of OVA shifted from 1645 to 1651 cm^{-1} and 1544 to 1548 cm^{-1} , respectively. These shifts clearly indicate the conformational change of OVA upon the formation of OVA NPs.

Table 4.1. Characteristics of nanoparticles in 1xPBS, pH 7.4 containing 0.1% PEG at 25 °C. Data are expressed as the mean $\pm$ SD ($n = 3$).

sample	size (nm)	polydispersity index	ζ -potential (mV)
OVA NP	91.2 $\pm$ 0.91	0.26 $\pm$ 0.01	-12.70 $\pm$ 0.86
OVA/RA NP	95.0 $\pm$ 2.63	0.27 $\pm$ 0.01	-12.53 $\pm$ 0.98
OVA/RA NP (reconstituted)	95.2 $\pm$ 0.39	0.26 $\pm$ 0.00	-10.3 $\pm$ 0.43

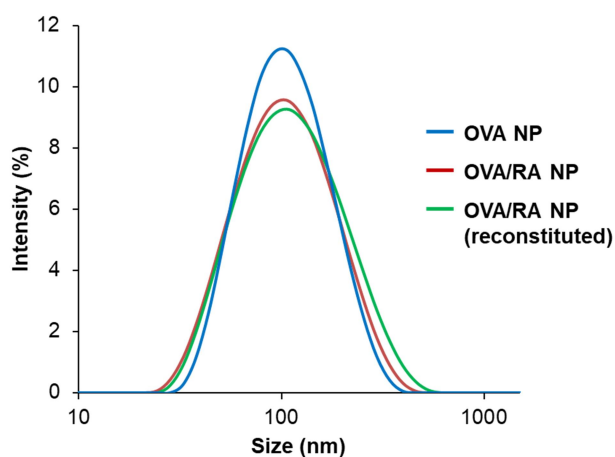


Figure 4.2 Size distribution of each nanoparticle in 1xPBS, pH 7.4 containing 0.1% PEG at 25°C.

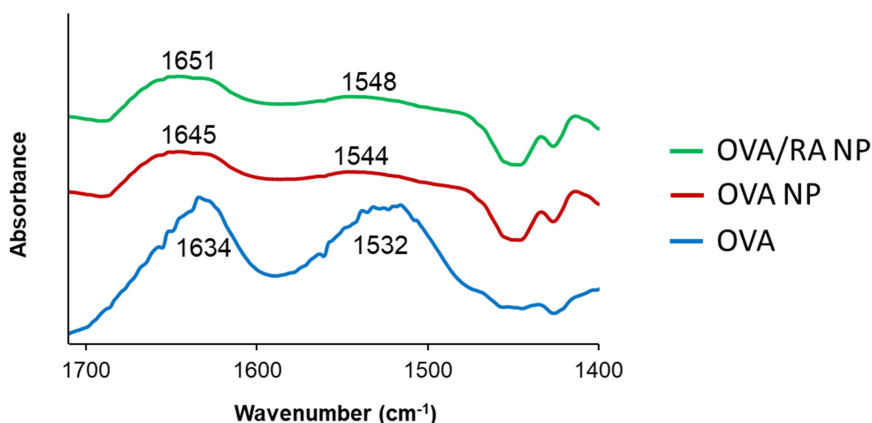


Figure 4.3. FT-IR spectra of OVA, OVA NP, and OVA/RA NP.

Then I examined complex formation of OVA NPs with ATRA. The binding of ATRA to OVA NPs was evaluated by quenching of intrinsic fluorescence of Trp residues of OVA upon binding with ATRA [22, 23]. Figure 4.4 A-B show changes of fluorescence spectra of native OVA and OVA NPs with increasing concentrations of ATRA, respectively. The increase in the ATRA concentration resulted in a gradual decrease in the fluorescence intensity of Trp residues without a shift of the maximum emission wavelength in both OVA and OVA NPs. These results were expressed as Hill plot in Figure 4.4 C. The K_d and n values determined from a linear fitting of Hill plot are summarized in Table 4.2. OVA NPs show somewhat higher affinity to ATRA than native OVA, while the n value was almost same in the both cases (~ 0.9). The dominant interaction between OVA NP and ATRA will be hydrophobic interaction. The similar binding characteristics between OVA NP and native OVA may indicate that a binding site of ATRA in OVA is conserved in OVA NP. The size distribution and the ζ -potential of the complex between OVA NP and ATRA (OVA/RA NP) prepared at OVA:ATRA ratio of 1:1 are shown in Figure 4.2A and Table 4.1. The size distribution and the ζ -potential of OVA/RA NP were almost same with original OVA NP. Effect of ATRA-binding to OVA NPs on the conformation of OVA was evaluated by FT-IR spectra (Figure 4.3). Amide I and amide II bands of OVA NPs were somewhat shifted after binding with ATRA, indicating the conformational change of OVA unit in OVA NP.

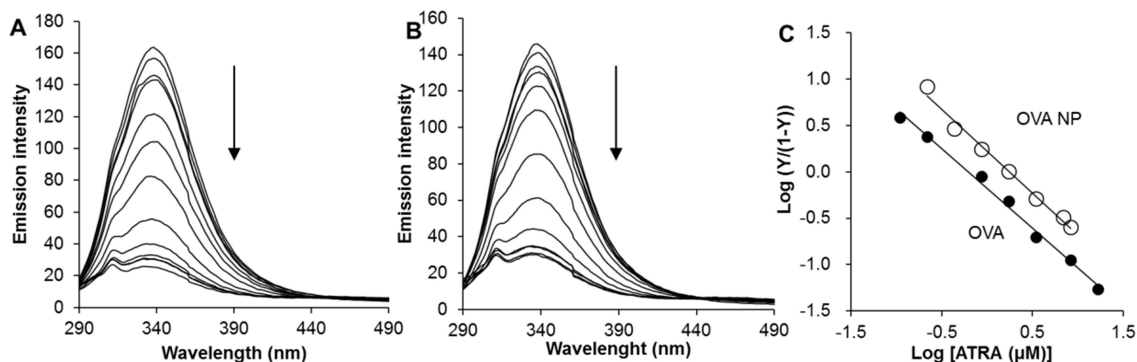


Figure 4.4 Fluorescence spectra of native ovalbumin (A) and OVA NPs (B) with increasing ATRA concentration in 1xPBS, pH 7.4 containing 0.1% PEG at 25 °C. [OVA] = [OVA NP] = 0.96 μ M-in-ovalbumin; [ATRA] = 0 to 27.7 μ M; λ_{ex} = 280 nm. (C) Hill plots of the results of panel A and B.

Table 4.2. Binding parameters for the interaction between ATRA and ovalbumin or OVA NP in 1xPBS, pH 7.4 containing 0.1% PEG at 25 °C. Data are expressed as the mean $\pm$ SD ($n = 3$).

sample	K_d (μ M)	n
OVA	1.105 ± 0.062	0.909 ± 0.045
OVA NP	0.739 ± 0.050	0.917 ± 0.021

Then, I tried to establish freeze-drying process of OVA/RA NPs. Dry formulation enables long time storage and is suitable for pulmonary administration via inhalation to treat asthma. To avoid aggregation of OVA/RA NPs upon freeze-drying, I used trehalose as a cryoprotectant which is approved for pulmonary administration [21, 24]. Figure 4.5 shows SEM image of the freeze-dried OVA/RA NPs. Particles with the size of 100 nm to a few hundred nm were observed. The dried sample was found to be re-dispersed rapidly in aqueous media (50 mM NaCl, pH 7) and the size of the reconstituted OVA/RA NPs did not change from the original ones. The rapid dissolving of the freeze-dried sample indicates that

the sample is suitable for pulmonary administration because it will be rapidly dissolved upon encountering the respiratory environment thereby releasing OVA/RA NPs.

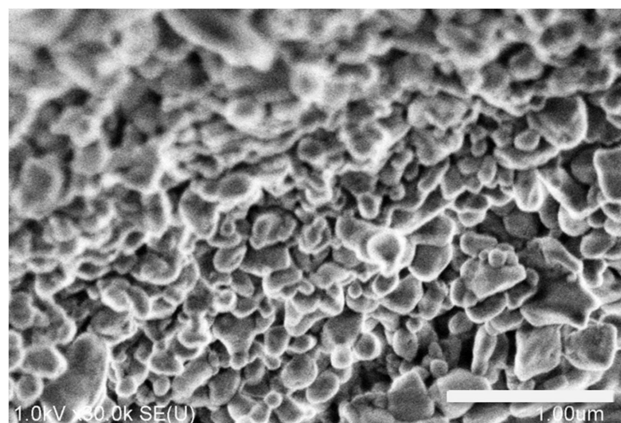


Figure 4.5 SEM image of freeze-dried OVA/RA NPs. Scale bar: 1.0 μm .

4.2.2 Induction of semimature DCs by OVA/RA NPs

First I examined cellular uptake of OVA NPs by cell line of DCs (DC 2.4). OVA NPs complexed with fluorophore DiO was used here. These complexes were prepared similarly with OVA/RA NPs. As shown in Figure 4.6, OVA NPs were engulfed by DC 2.4 with time and localized in cytosol. Dendritic cells are professional phagocytes, which can engulf outer nanoparticles regardless of their surface characteristics. Thus, OVA NPs which have negative surface charge can be engulfed by DC 2.4.

Then, I examined induction of semimature state of DCs by OVA/RA NPs. In the semimature state of DCs, presentation of antigen peptides via MHC class II is somewhat activated while expression of co-stimulatory receptors CD80/86 is kept as low as immature DCs [7, 8, 25, 26]. As shown in Figure 4.7 A, DC 2.4 treated with OVA/RA NPs showed slightly higher expression of MHC class II than other control groups. Although ATRA was reported to slightly raise the expression of MHC class II in DCs [27], it has not been clarified the reason why the expression of MHC class II was activated only in the case of OVA/RA NPs. As for the co-stimulatory receptors CD80/86, their expression levels after treatment with OVA/RA NPs were as same as those of non-treated immature DC 2.4 (Figure 4.7 B). Taken together, OVA/RA NPs successfully induced semimature state in DC 2.4.

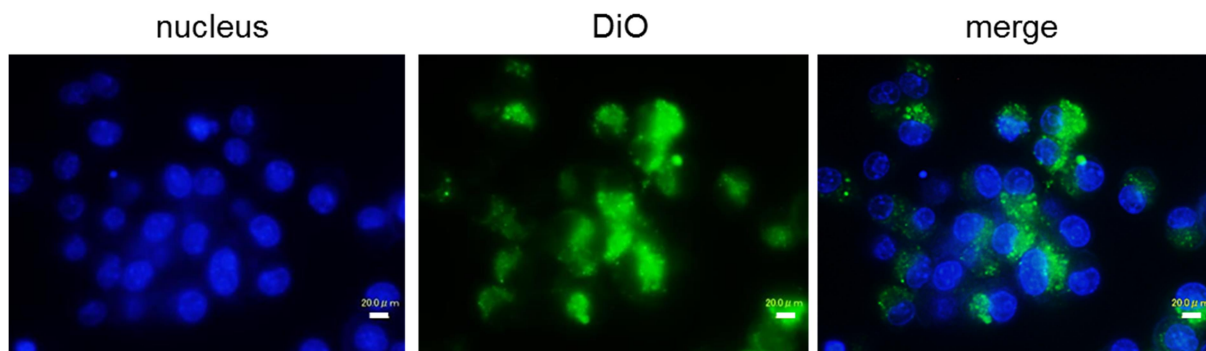


Figure 4.6 Cellular uptake of OVA NPs by DC 2.4 cells after 6 h incubation. OVA NPs were labeled with DiO. Scale bar: 20 μ m.

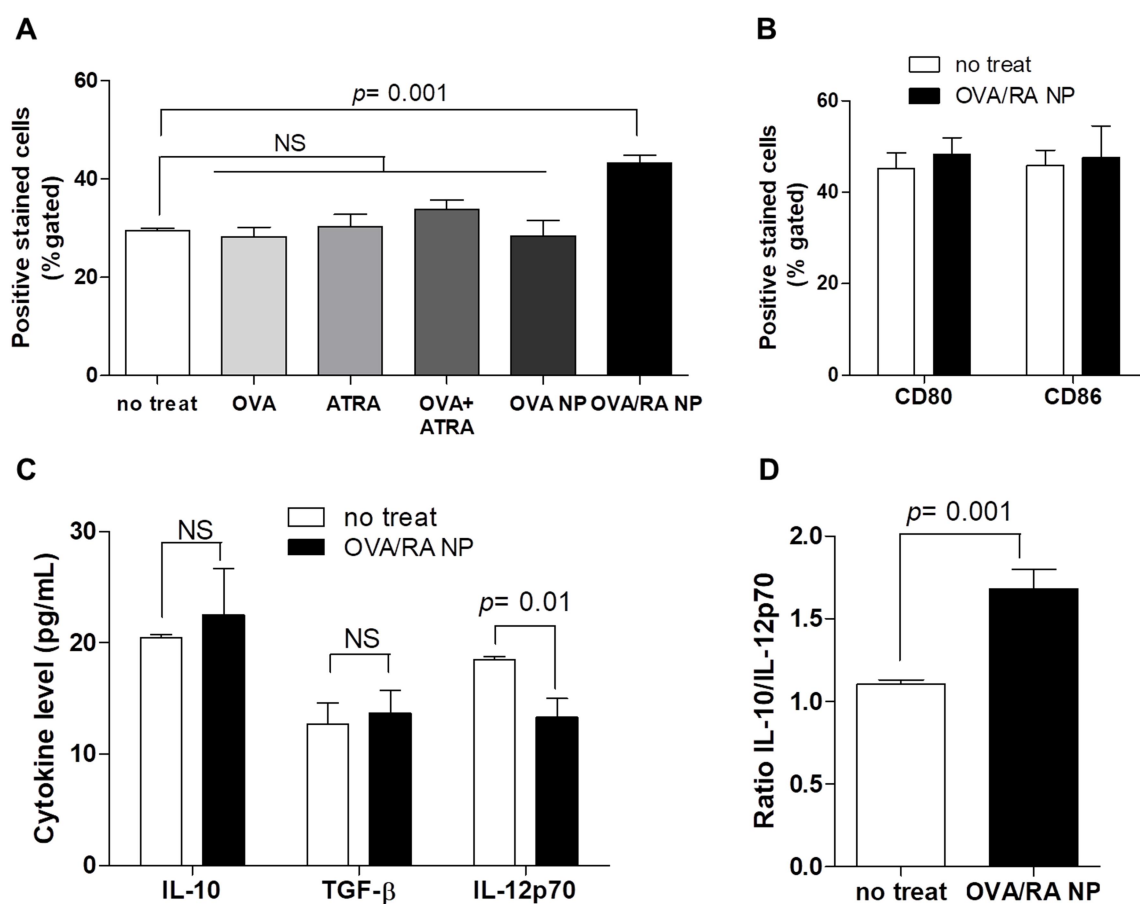


Figure 4.7 Expression of MHC class II (A) and co-stimulatory receptors CD80/86 (B) in DC 2.4. Secretion of cytokines from DC 2.4 treated with OVA/RA NPs (C) and ratio of concentration of IL-10/IL-12p70 (D). DC 2.4 cells were treated with each sample (conc. of ovalbumin and ATRA: 0.1 μ M) for 24 h at 37°C. NS; not significant.

I also examined secretion of cytokines from DC 2.4 after the treatment with OVA/RA NPs. As shown in Figure 4.7 C, the secretion of inflammatory cytokine IL-12p70 was suppressed by OVA/RA NPs comparing with the non-treated immature DC 2.4, while the secretion of anti-inflammatory cytokines IL-10 and TGF- β was kept unchanged. As the result, ratio of expression of IL-10/IL-12p70, which is used as a measure of anti-inflammatory response of cells [26-28] was raised by OVA/RA NPs (Figure 4.7 D). These signatures of DC 2.4 induced by OVA/RA NPs are expected to promote differentiation of naïve T cell to Treg [31, 32] which will result in protection from antigen-specific immune response.

4.3 Summary

Here I established the preparation procedure of the complex between OVA NPs and ATRA. The obtained OVA/RA NPs successfully enhanced semimatururation of DCs, i.e., negligible effect on the expression of co-stimulatory receptors while weakly upregulating expression of MHC class II, and raising IL-10/IL-12p70 ratio (Figure 4.7 D and Figure 4.8). The formulation OVA/RA NPs will not be limited to OVA but generally applicable to any antigen proteins with hydrophobic pockets. The present study would open up new technological and pharmaceutical innovations for antigen-specific immunotolerance therapy.

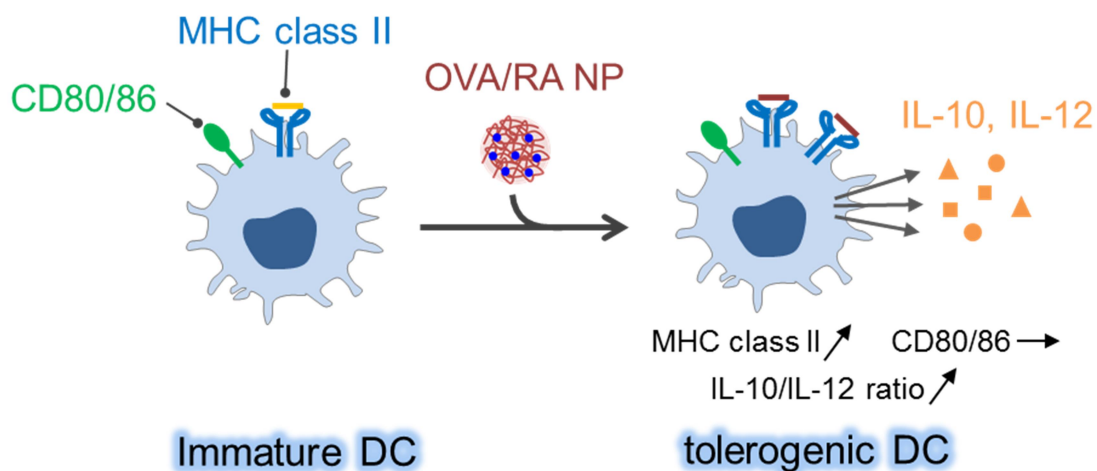


Figure 4.8 Induction of tolerogenic DC by OVA/RA NP

4.4 Experimental section

Materials

Ovalbumin (OVA), trehalose, and polyethylene glycol (PEG, Mw 4600) were purchased from Sigma. All-trans retinoic acid (ATRA) was purchased from TCI. 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from Takara-Clontech. Gibco™ 10xPBS (Phosphate Buffered Saline), pH 7.4 was purchase from Thermo fisher scientific.

Preparation of ovalbumin nanoparticle

OVA nanoparticles (OVA NPs) were prepared by following the method reported previously [16]. OVA was dissolved in 50 mM NaCl aqueous solution to be 10 mg/mL (0.24 mM), then pH of the solution was adjusted by 3M NaOH to be pH 7.0. 2.0-mL aliquot of the solution was placed in a glass tube and the solution was heated in a water bath at 85°C for 5 min, then immediately cooled in an ice bath for 10 min. The dispersion of OVA NPs thus obtained was kept at 4°C until use.

Preparation of complex between OVA NPs and ATRA (OVA/RA NPs)

By using the dispersion of OVA NPs, a dispersion containing 27.2 μ M OVA NPs, 0.1% PEG, and 1xPBS, pH 7.4 (Gibco) was prepared. To this dispersion, ethanolic solution of ATRA (11.1 mM) was added to achieve desired molar ratio of OVA and ATRA. Final ethanol concentration in the mixture was less than 1 vol% to avoid structural change of OVA in OVA NPs. The resulting dispersions were placed in darkness for 3 h for equilibration to form OVA/RA NPs. For freeze drying of the dispersion of OVA/RA NP, trehalose powder (Sigma) was added to the dispersion to be the final concentration of 20 mg/mL, then the resulting dispersion was freeze dried by using FDU-2100 (EYELA, Japan) for 3 days. The obtained freeze dried OVA/RA NPs were used for in vitro study. Complex between OVA NPs with fluorescent DiO was prepared similarly.

Measurement of size distribution and ζ -potential of nanoparticles

Dispersion containing each nanoparticle (4.75 μ M in OVA conc.) and 0.1% PEG in 1xPBS, pH 7.4 (Gibco) was prepared. The size, polydispersity index and ζ -potential of nanoparticles were measured with Zetasizer Nano (Malvern Instruments, UK) at 25 °C.

Determination of binding parameters

In order to determine binding stoichiometry (n) and dissociation constant (K_d) between ATRA and OVA, quenching of intrinsic fluorescence of OVA by ATRA was used [17]. Samples were prepared by adding ethanolic solution of ATRA (11.1 mM) to 1.0 ml of solution containing 0.96 μ M OVA or OVA NP, 0.1% PEG, and 1xPBS, pH 7.4 (Gibco). The resulting solution was placed in darkness for 3 h at 25°C for reaching equilibrium. The emission spectra of the resulting solutions were recorded by using Jasco Spectrofluorometer FP-8600 ($\lambda_{\text{ex}} = 280$ nm). Fluorescence quenching was analyzed using the modified Hill equation:

$$\text{Log } \frac{Y}{(1-Y)} = n \cdot \text{Log } C - \text{Log } K_d$$

where Y is the relative fluorescence in the presence of quencher (ATRA), n is the number of binding sites of ATRA per one OVA, C is the concentration of ATRA, and K_d is the dissociation constant. The plot of $\text{Log } [Y/(1-Y)]$ versus $\text{Log } C$ yields $\text{log } K_d$ as the intercept, and n as the slope. From the intercept and slope, the n and K_d values were calculated.

FT-IR measurements

Universal attenuated total reflectance (UATR) measurements of freeze-dried nanoparticles were carried out with a Spectrum Two FT-IR Spectrometer (PerkinElmer).

Scanning electron microscopy

Morphology of the freeze-dried nanoparticles was observed by using a Hitachi SU8000. Samples were mounted on an aluminum stub with the aid of adhesive carbon tape.

Cell culture

DC 2.4 cells were maintained in complete RPMI medium containing 10% fetal bovine serum (FBS) and 4 mM L-glutamine supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.05 mM β -mercaptoethanol, and MEM non-essential amino acid solution. The cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

Cellular uptake

DC 2.4 cells (1×10^4 cells/well) were seeded in a 96-well glass surface plate with complete RPMI. After 24 h of incubation, the cells were washed twice with 1x DPBS.

Complete RPMI containing 1.1 μM OVA NP complexed with DiO was added to the well. After 6 h of incubation at 37°C, the cells were observed by fluorescence microscopy.

Induction of tolerogenic dendritic cell

DC 2.4 cells (1×10^5 cells) were seeded in a 6-well plate with complete RPMI. After 24 h of incubation, the medium was replaced with RPMI complete medium containing various samples (in all the samples, concentrations of OVA and ATRA were 0.1 μM), then the cells were incubated for 24 h. The supernatant of the cell culture was collected for quantification of cytokines (TGF- β , IL-10, IL-12p70) by ELISA following the manufacturer's protocol (R&D systems). The cells were collected by scrapping and resuspended in 400 μL medium. Subsequently, 5 μL of 7-AAD and 5 μL of anti-mouse MHC class II (I-A/I-E) PE (clone: M5/114.15.2); 5 μL of propidium iodide and anti-mouse CD80 (clone: 16-10A1) or anti-mouse CD86 (clone: PO3) and were added and incubated for 30 min at 4°C. The resulting cells were analyzed by flow cytometry by using Sony EC800 instrument.

3.5 References

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CHAPTER 5

Conclusions

Thanks to the characteristics of nanomedicine that lipid-based nanoparticle and protein-based nanoparticle have, developing strategies to design tolerogenic nanoparticle have been extensively evolved. Whether encapsulation or conjugation of immunomodulation molecules with nanoparticles is through non-covalent, covalent or physical interaction, the ultimate purposes are to improve efficacy of existing drugs and develop new therapeutic systems. Nanoparticle therapies are growing and used in the understanding of the underlying pathogenesis of immune-mediated diseases. Nanoparticles also have potential to restore peripheral immune tolerance or to regulate aberrant monocyte activities during severe inflammation. However, the functional outcome of nanoparticles depends on many intrinsic particle factors and also is interfered by external factor such as route of administration. To address this issue and to utilize the potential of nanoparticle, development of an inert and simpler nanoparticle is required. In this thesis, I approached this goal through two methods.

In chapter 2, I could successfully prepare effective formulation of nanostructured lipid carrier (NLC) for delivery of $1,25(\text{OH})_2\text{D}_3$ to colonic inflammatory lesions via oral administration. NLC as lipid-based nanoparticle is suitable for incorporation of hydrophobic molecules. NLC orally delivered $1,25(\text{OH})_2\text{D}_3$ to the colon in mice and maintained a high concentration of $1,25(\text{OH})_2\text{D}_3$ in the colonic tissue for at least 12 h. The NLC showed multiple effects on the suppression of symptoms of colitis induced by dextran sodium sulfate, namely maintaining crypt structure, reducing the tissue concentration of inflammatory cytokines, suppressing the infiltration of polymorphonuclear leukocytes, and augmenting anti-inflammatory $\text{CX}_3\text{CR1}^{\text{high}}$ macrophages. My NLCs containing $1,25(\text{OH})_2\text{D}_3$ may be an alternative treatment for IBD therapy.

In chapter 3, I followed the first strategy which uses NLC as carrier for regulation inflammation. I used the same formulation of NLC for incorporation and delivery of all-trans retinoic acid. NLC containing retinoic acid (NLC-RA) thus obtained was taken up by macrophages and induced anti-inflammatory response via suppressing NF- κ B signaling as well as via enhancing the production of anti-inflammatory cytokines. Moreover, NLC-RA enhanced differentiation of naïve T cells to regulatory T cells in the co-culture system with

dendritic cells. These results suggest that NLC-RA is a promising alternative therapy for the autoimmune diseases especially intestinal bowel disease.

In chapter 4, I proposed a simple formulation of ovalbumin nanoparticles complexed with all-trans retinoic acid (OVA/RA NPs) induced phenotype of tolerogenic dendritic cells (tDCs). tDCs play a crucial role in the immunotolerance therapy because they induce anergic response for auto-reactive helper T cells and also enhance differentiation to regulatory T cells to maintain tolerance against auto-antigens. All-trans retinoic acid (ATRA) is one of the representative molecules to induce tDCs. As a result, OVA/RA NPs were taken up by DCs and successfully induced phenotype of tolerogenic dendritic cells (tDCs). These promising features of OVA/RA NPs will be suited for immunotolerance therapy and may be applicable to pulmonary delivery via inhalation to treat allergic asthma.

Regarding the result of Chapter 2 and Chapter 3, I expect that both systems could be merged as NLC containing vitamin D₃ and retinoic acid. This system may be more powerful, resulting in modulation of many pathways for IBD treatment. For Chapter 4, it still needs extended experiment to evaluate the particle effect on allergic mice model.

The novel approaches and findings in this research may provide new insights into the development of more straightforward methods to utilize the unique advantages of nanoparticles in immunotolerance therapy. And in a broader sense, they can lead realizing of more efficient and cheaper medications for severe diseases.

Achievements

List of publications

1. **Khadijah zai**, Masahiro Hirota, Takahiro Yamada, Narumi Ishihara, Koichiro Suzuki, Takeshi Mori, Akihiro Kishimura, Koji Hase, and Yoshiki Katayama, “Therapeutic effect of vitamin D3-containing nanostructured lipid carriers on inflammatory bowel disease”, *Journal of Controlled Release*, 2018, *press*.
2. **Khadijah zai**, Narumi Ishihara, Hiroyuki Oguchi, Masato Hirota, Akihiro Kishimura, Takeshi Mori, Koji Hase, and Yoshiki Katayama, “Regulation of inflammatory response of macrophages and induction of regulatory T cells by using retinoic acid-loaded nanostructured lipid carrier”, *Journal of Biomaterials Science: Polymer Edition*, 2018, *press*.
3. **Khadijah Zai**, Kazuki Yuzuriha, Akihiro Kishimura, Takeshi Mori, and Yoshiki Katayama, “Preparation of complexes between ovalbumin nanoparticles and retinoic acid for efficient induction of tolerogenic dendritic cells”, *Journal of Analytical Sciences*, 2018, *press*.

List of supplementary publications

1. Elnaz Nakhaei, Ko Takehara, Hikari Sato, **Khadijah Zai**, Akihiro Kishimura, Takeshi Mori and Yoshiki Katayama, “A Dual Alkylated Peptide-ligand Enhances Affinity to Human Serum Albumin”, *Journal of Analytical Sciences*, 2018, 34, 501-504, [selected as Hot Article].
2. Md. Zahangir Hosain, Kazuki Yuzuriha, **Khadijah Zai**, Masafumi Takeo, Akihiro Kishimura, Yoshihiko Murakami, Takeshi Mori, and Yoshiki Katayama, “Synergic modulation of the inflammatory state of macrophages utilizing anti-oxidant and phosphatidylserine-containing polymer-lipid hybrid nanoparticles”, *Journal of MedChemComm*, 2017, 8, 1514-1520.

List of presentations

1. “Resolution of Intestinal Bowel disease by Using Nanostructure Lipid Carrier Containing Nuclear Receptor Agonist”, Poster presentation in 7th Annual Meeting of the Japan Biomaterials Association, Kyushu, Japan, December 8, 2017.
2. “Resolution of Intestinal Bowel disease by Using Nanostructure Lipid Carrier Containing Nuclear Receptor Agonist”, Oral presentation in 1st Student Seminar of International Graduate Course on Chemistry for Molecular Systems, Kyushu, Japan, May 18, 2017, [selected as Best presenter].

Acknowledgments

Completion of this research work and dissertation could not be achieved without the assistance, cooperation and understanding of multiple people.

First and foremost, I wish to thank my supervisor Professor Yoshiki Katayama for his sustained support during the three years. Under his guidance and advisory I was able to solve difficultness that I was facing during my experimental and research path. To me, his constant understanding and back up was always the greatest motivation to be able to keep doing my best and going forward.

Special mention goes to my research advisor, Associate Professor Takeshi Mori. It has been an honor to be his first Ph.D. student from Immunity group. He has taught me, both consciously and unconsciously, how to be good researcher. I appreciate all his contributions of time and ideas to make my Ph.D. experience productive and stimulating. I am thankful to him as he patiently taught me the correct way of making advanced presentations or writing decent scientific papers, which were accompanied with reminding me many subtle yet important points.

I would like to express my sincere thanks to Associate Professor Akihiro Kishimura for his valuable advice on my experimental procedures during discussions and presentations.

I am deeply grateful for the collaborative assistance of Professor Koji Hase and also his students (Mr. Masato Hirota, Mr. Takahiro Yamada, Ms. Narumi Ishihara, and Mr. Hiroyuki Oguchi). Professor Koji Hase generously provided me with his methodological knowledge and Prof. Hase's team supported me in animal experiment, which was a critical part in my research hypothesis.

I would also like to express my special thanks to my research fellows, Immunity group, Kazuki Yuzuriha, Yunmei Mu, Ayaka Yoshida, Haruka Nagai, and Li Shunyi for the discussion times that I had over my research ideas.

My sincere thanks to all my doctoral thesis committee members, Professor Masahiro Goto and Professor Yoshiko Miura for their time and invaluable advice on my thesis project.

My fruitful educational stay in the prestigious Kyushu University and Katayama laboratory could not be realized without the financial support from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), which I am greatly appreciative.

Lastly, I would like to thank my family for all their love and encouragement. For my parents who raised me with a love and supported me in all my pursuits. And also my brother-sisters for their compassionate support throughout my study in abroad.

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September, 2018