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Therapeutic effect of vitamin D_3-containing nanostructured lipid carriers on inflammatory bowel disease

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ABSTRACT

The active form of vitamin D_3, $1,25(OH)_2D_3$ has been found to exert multiple effects on the suppression of progression of inflammatory bowel disease (IBD). Vitamin D_3 has been gathering attention as a therapy for IBD. However, the clinical trials conducted to date revealed that a relatively high dosage of vitamin D_3 was required to see a significant therapeutic effect. Thus, effective formulation and delivery of vitamin D_3 to colonic inflammatory lesions will be required. Herein we describe the preparation of a nanostructured lipid carrier (NLC) for the encapsulation of $1,25(OH)_2D_3$ for colonic delivery via oral administration. The optimized fabrication procedure enabled the incorporation of $1,25(OH)_2D_3$ in the NLC by minimizing the destruction of chemically unstable $1,25(OH)_2D_3$. The obtained NLCs orally delivered $1,25(OH)_2D_3$ to the colon in mice and maintained a high concentration of $1,25(OH)_2D_3$ 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 CX_3CR1^{high} macrophages. Our NLCs containing $1,25(OH)_2D_3$ may be an alternative treatment for IBD therapy.

Keywords:
Inflammatory bowel disease
Vitamin D_3
Oral administration
Inflammatory disease
Nanostructured lipid carrier

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 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 D_3 is a pleiotropic hormone that conventionally controls calcium homeostasis and bone mineralization [8]. Recently, a modulatory role of vitamin D_3 on the activity of immune cells has received attention [9,10]. Vitamin D_3 is absorbed from the diet and synthesized in the skin during sun exposure. Vitamin D_3 is converted to $25(OH)D_3$ and then $1,25(OH)_2D_3$ by different enzymes existing in many kinds of cells, especially liver, kidney, and immune cells [8-10]. $1,25(OH)_2D_3$, which is the active form of vitamin D_3, is an agonist of the vitamin D_3 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 deficiency of $25(OH)D_3$ and $1,25(OH)_2D_3$ in their serum, a vitamin D_3 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 D_3 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 D_3 toward IBD are advantageous compared with the presently available drugs that work to regulate the inflammatory response of immune cells. Thus, vitamin D_3 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 D_3 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 D_3 much higher than the conventionally accepted dosage was required [23]. A high dosage of vitamin D_3 is known to produce

adverse effects such as hypercalcemia [20]. Thus, effective delivery of vitamin D_3 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 D_3 to the inflammatory lesions of IBD model mice. Froicu and Cantorna reported that rectal administration of $1,25(OH)_2D_3$ showed a better therapeutic effect than oral administration because rectal administration can directly deliver $1,25(OH)_2D_3$ 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 D_3 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 D_3 [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, we tried to develop NLCs to deliver $1,25(OH)_2D_3$ 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, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$. To avoid the destruction of $1,25(OH)_2D_3$ during NLC preparation, we developed a new fabrication procedure for NLCs containing $1,25(OH)_2D_3$. The NLCs thus obtained were orally administered to mice. We found that the NLCs maintained a high concentration of $1,25(OH)_2D_3$ in colonic tissue for a relatively long time and significantly suppressed the progression of disease in IBD model mice.

2. Materials and Methods

2.1. Materials

L-α Phosphatidylcholine (PC) from soybean, Pluronic F-127 and α-tocopherol were purchased from Sigma-Aldrich. $1,25(OH)_2D_3$ 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'-Dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from Takara-Clontech.

2.2. Preparation and analysis of NLC

2.2.1. Preparation of the NLCs

NLCs containing $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (20 μg/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 μg/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.

2.2.2. 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.

2.2.3. 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.

2.2.4. Determination of the entrapment efficiency of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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.

2.3. *In vitro* study

2.3.1. 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% CO_2 and 95% air at 37°C.

2.3.2. Cellular uptake of NLC

RAW 264.7 macrophages (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 0.01% 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.

2.3.3. 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).

2.3.4. NF-κB-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).

2.3.5. 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 ΔΔCt 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 Supplementary Table S1.

2.4. *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 $1,25(OH)_2D_3$), or NLC-D3 (0.262 μg/ml of $1,25(OH)_2D_3$; 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. $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ in the tissue extracts were measured using the

$1,25(OH)_2D_3$ EIA kit following the manufacturer's protocol (Immunodiagnostics Systems, Scottsdale, AZ).

2.5. NLC therapy *in vivo*

2.5.1. 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 $1,25(OH)_2D_3$), or NLC-D3 (0.262 μg/ml of $1,25(OH)_2D_3$; 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).

2.5.2. 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.

2.5.3. 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×*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 µg/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.

2.5.4. 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-CX_3CR1 (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).

2.5.5. 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 ΔΔCt 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 Supplementary Table S2.

2.5.6. Statistical analysis

Data were analyzed using R version 3.4.2. The data were initially tested with Bartlett's test for equal variances. Following that, the data were subjected to either the non-parametric Kruskal–Wallis followed by Steel's test or the parametric one-way analysis of variance (ANOVA) followed by Dunnett's test with The R package multcomp [39]. A P value of less than 0.05 was considered to be statistically significant. Data are presented as the mean $\pm$ SD.

3. Results and discussion

3.1. 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 D_3 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 $1,25(OH)_2D_3$ to prepare the pre-emulsion at ambient temperature. As the core components of the NLCs, we selected GMS and α-tocopherol as the solid and liquid lipid, respectively. α-Tocopherol is a representative antioxidant and is expected to protect $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ from radical species, we added ascorbic acid in the aqueous phase [42]. As surfactants to stabilize the NLC, a combination of a neutral polymer (Pluronic F127) and phospholipids (PC: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 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (run 5). The obtained NLCs were confirmed to be a spherical shape by TEM (Fig. S1 A). We concluded that the preparation condition used for run 5 was the best among the ones we examined. Without ascorbic acid in the aqueous phase, the content of the intact $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 (Fig. S1B). Table 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 1. Characteristics of NLC-D3 prepared under various conditions. Data are expressed as mean ± SD (n = 3).

run	condition		size (nm)	polydispersity index	$1,25(OH)_2D_3$ in NLC (μg)	$1,25(OH)_2D_3$ in aq. phase (μg)	entrapment efficacy (%)
	homogenization	ultrasonication					
1	15 min	-	274 ± 28	0.74 ± 0.12	12.53 ± 0.55	3.31 ± 0.49	62.7 ± 2.7
2[a]	-	10%, 5 min	-	-	-	-	-
3	-	10%, 15 min	597 ± 128	0.72 ± 0.16	2.66 ± 0.41	0.73 ± 0.64	13.3 ± 2.1
4	-	30%, 5 min	116 ± 2	0.33 ± 0.01	5.76 ± 0.88	0.27 ± 0.24	28.8 ± 4.4
5	15 min	10%, 5 min	110 ± 4	0.23 ± 0.01	12.57 ± 0.64	1.32 ± 0.60	62.8 ± 3.2

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

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

name	content	size (nm)	polydispersity index	ζ-potential (mV)
NLC	-	108 ± 6	0.23 ± 0.02	-17.60 ± 0.96
NLC-D3	$1,25(OH)_2D_3$	110 ± 4	0.23 ± 0.01	-17.10 ± 0.30
NLC-DiO	DiO	106 ± 7	0.24 ± 0.00	-16.70 ± 0.90
NLC-DiO (-) PS[a]	DiO	171 ± 5	0.24 ± 0.00	-9.94± 0.37

[a] Prepared without adding PS.

3.2. Cellular uptake and cytotoxicity of NLCs toward macrophages

We 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 Fig. 1A, NLC-DiO (0.01%) was actively taken up by the macrophages after a 24 h incubation. Treatment of NLCs without PS resulted in a significant reduction in the cellular uptake (Fig. 1B), 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 % (Fig. 1C).

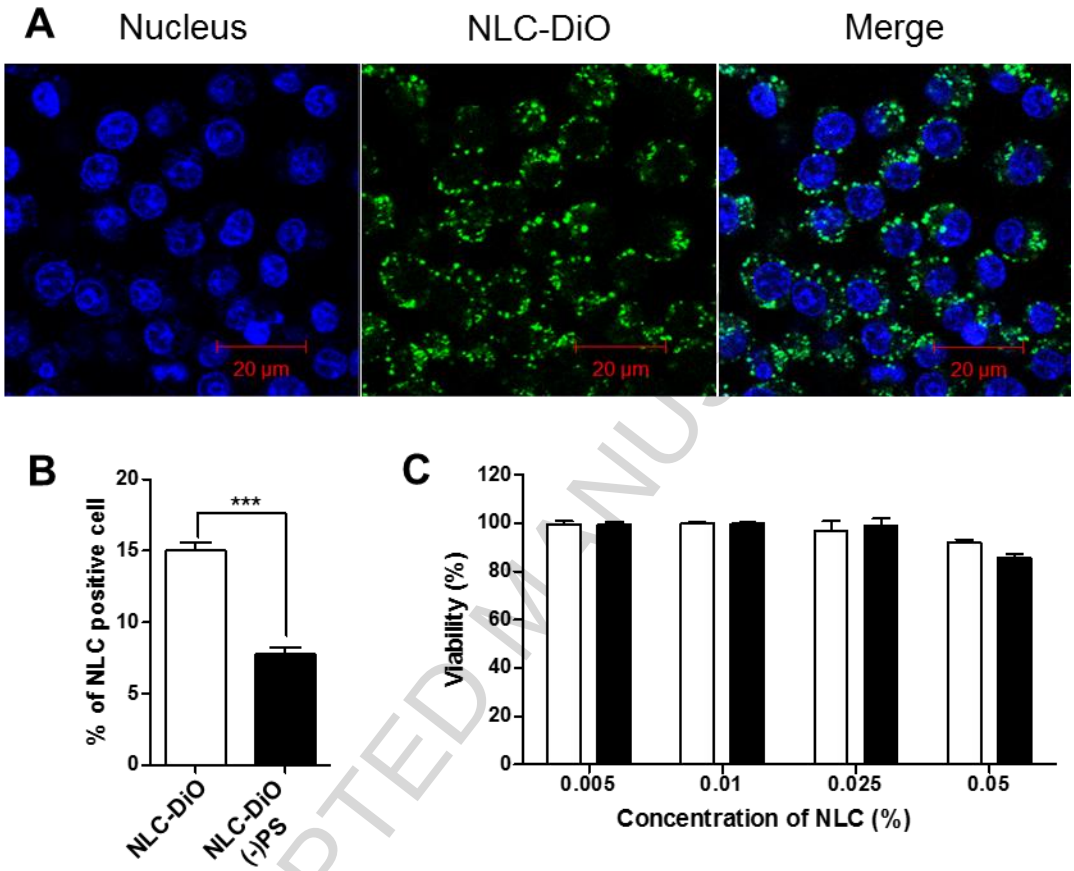

Fig. 1. (A) Cellular uptake of NLCs by RAW 264.7 macrophages after 24 h incubation. Blue and green colors represent the nucleus and NLC-DiO, respectively. (B) Cellular uptake of NLCs and PS-removed NLCs after 6 h incubation. (C) Viability of RAW 264.7 macrophages after treatment with empty NLCs (white 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 ± SD (n = 3). *** $P < 0.001$.

3.3. 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-κB dependent pathway [52]. Because $1,25(OH)_2D_3$ has been reported to suppress the inflammatory response in macrophages [53-55], we examined the suppressive effect of NLC-D3 in activation of the NF-κB pathway triggered by LPS. To this end, we 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-κB response element. As shown in Fig. 2A, not only NLC-D3 but also empty NLCs significantly suppressed the activation of the NF-κB 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-κB pathway, we 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-κB pathway by the empty NLCs resulted from the neutralization of ROS by α-tocopherol [40,41]. Hereafter, we used NLC-D3 containing both PS and α-tocopherol.

Next we 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-1β) compared to the empty NLCs. This result underscored the suppressive effect of $1,25(OH)_2D_3$ on the inflammatory response of the macrophages.

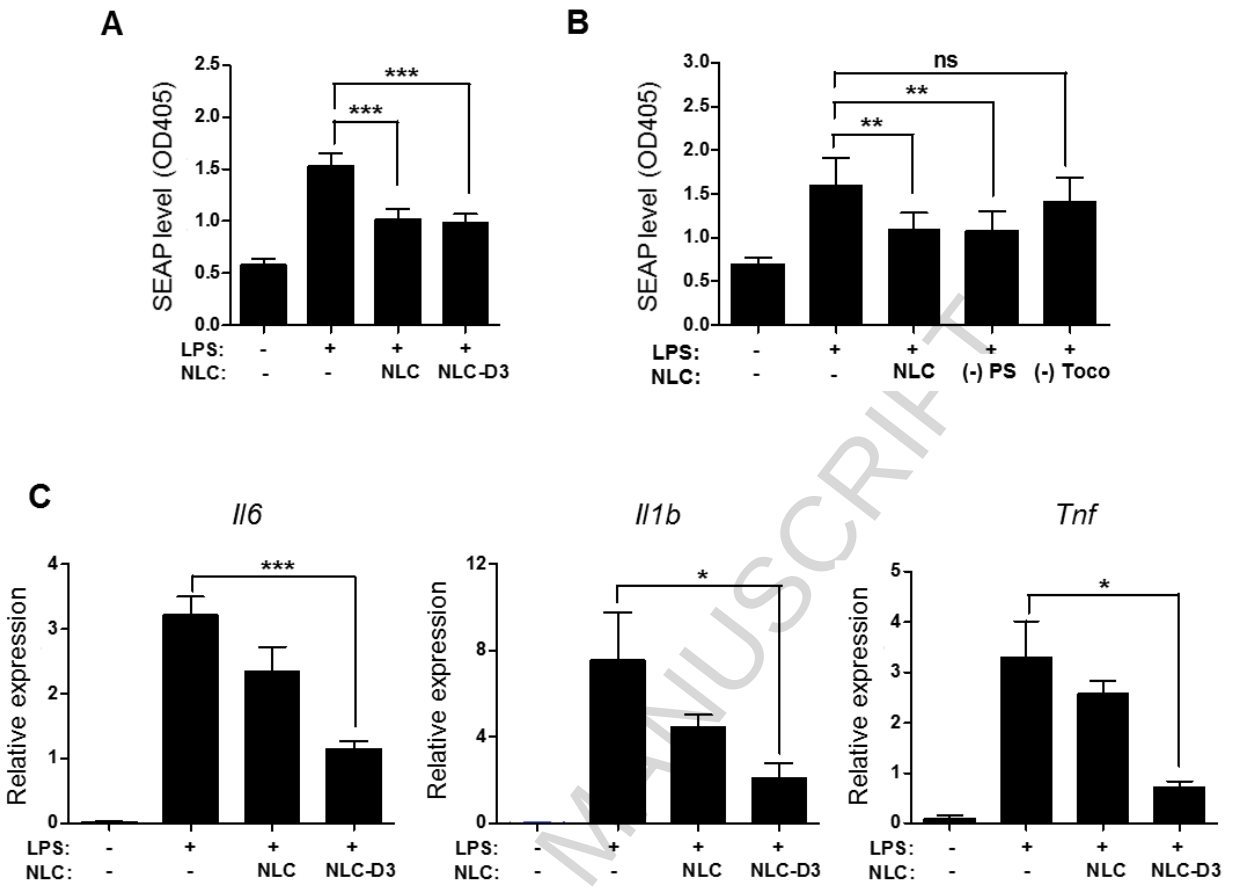

Fig. 2. (A, B) Suppressive effects of NLCs on the LPS-induced NF-κB activation in RAW264.7 macrophages. After addition of each NLC [0.01%, containing 8.49 nM of $1,25(OH)_2D_3$] to macrophages for 6 h, LPS (20 ng/mL) was added and incubated for a further 18 h. Absorbance resulting from the substrate 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 ± SD (n = 3). N.S., not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.4. NLC facilitated delivery of vitamin D3 to the colon

To evaluate the delivery efficiency of $1,25(OH)_2D_3$ into the colon by NLC-D3, we measured the concentration of $1,25(OH)_2D_3$ in the colonic tissue after gavage administration. As shown in Fig. 3, administration of $1,25(OH)_2D_3$ as a saline dispersion failed to increase $1,25(OH)_2D_3$ concentrations in the colon tissue. In contrast, NLC-D_3 significantly raised the colonic concentration of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ in NLC allowed sustained delivery of $1,25(OH)_2D_3$ to the colon for a long duration.

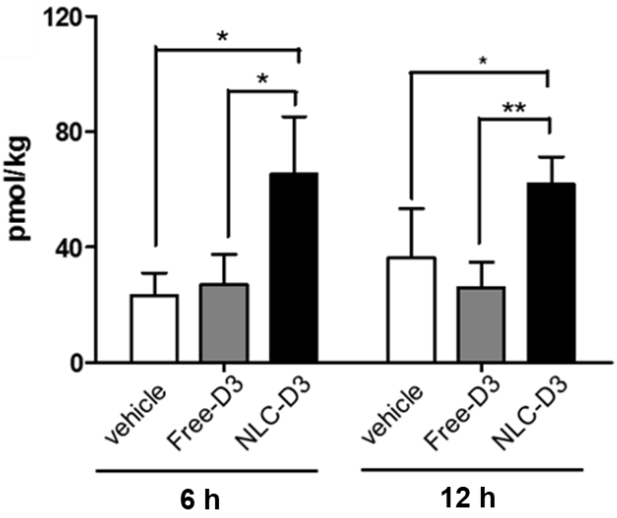

Fig. 3. $1,25(OH)_2D_3$ levels in colonic tissues after oral administration of vehicle, free-D3 or NLC-D3. Data are presented as the mean ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.5. NLC-D3 ameliorated symptoms of DSS-induced colitis

We 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 [56]. A dose of ca. 120 pmol/day of $1,25(OH)_2D_3$ was chosen for this study, because this does 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 (Fig. 4A). Treatment with NLC-D3 ameliorated the clinical symptoms from Day 3 onward, and decrease

in body weight was suppressed from Day 6 (Fig. 4B). 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. 4C, 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).

Proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, mediate development of IBD [5] as well as DSS-induced colitis [57]. We observed that expression of *Il6* was remarkably downregulated in the colonic tissue of mice treated with NLC-D3 but not free-D3 (Fig. 4E). 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 [56]. Treatment with NLC-D3 also prevented splenomegaly, suggesting attenuation of bacterial translocation (Fig. 4F, G). 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.

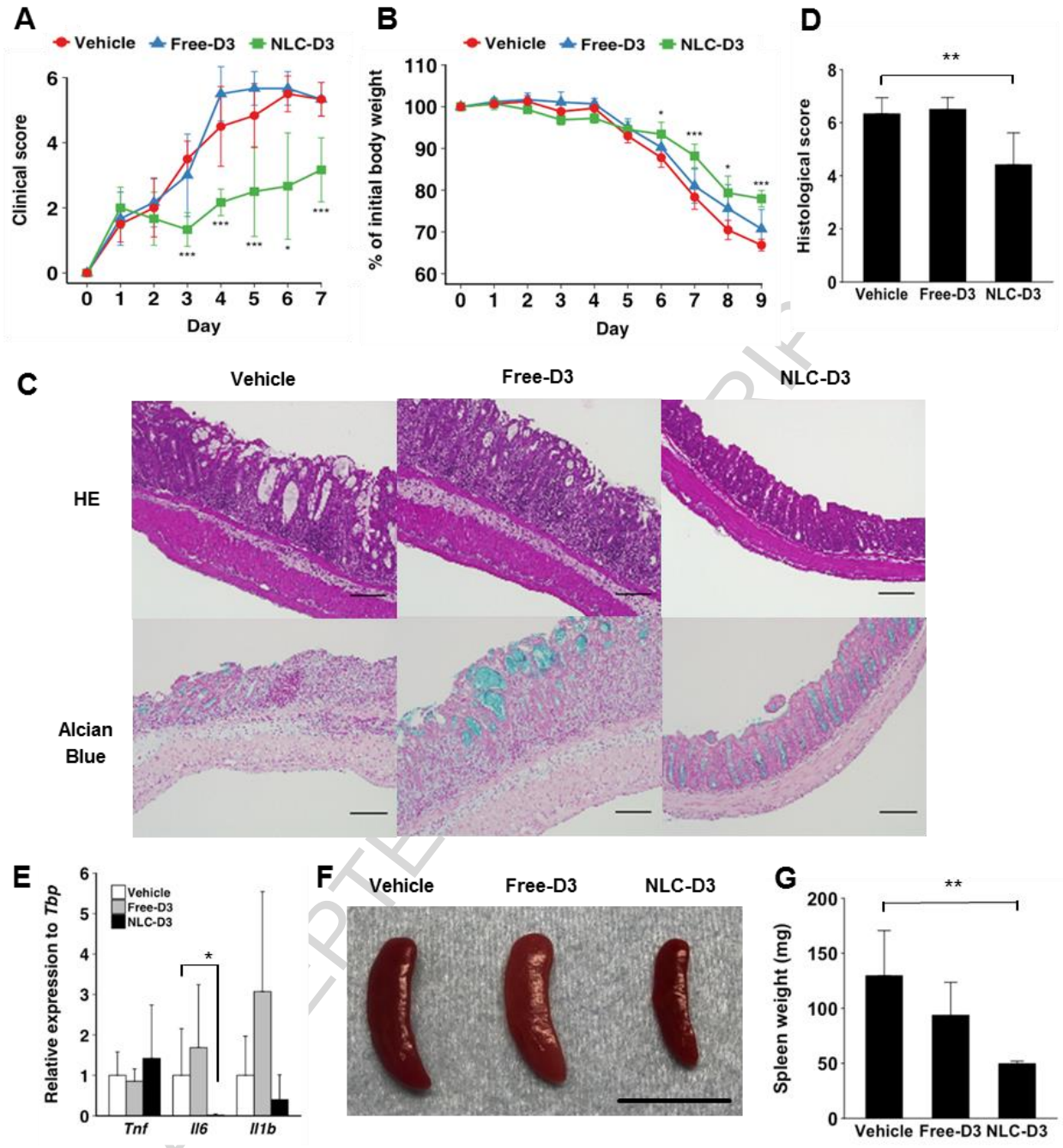

Fig. 4. Effects of NLC-D3 on dextran sodium sulfate-induced colitis. (A–G) Mice were administered 2% dextran sodium sulfate (DSS) for 6 days followed by normal drinking water. During the course of the experiment, the mice received oral gavage of the vehicle, free-D3, or NLC-D3 once a day from Day 0. 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 μm. 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 ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ vs vehicle control. Data are representative of two independent experiments.

3.6. 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, we analyzed the composition of the immune cells in the colonic lamina propria by flow cytometry. We found that administration of NLC-D3 diminished infiltration of polymorphonuclear leukocytes (PMN), namely eosinophils and neutrophils (Fig. 5A, B), whereas NLC-D3 treatment did not affect the frequency of the monocytes (Fig. 5C). NLC-D3 treatment slightly increased the frequency of total macrophages ($CD11b^{+}$MHC class II^{+}) although the difference was not statistically significant (Fig. 5D). Among the macrophage populations in the gut, the CX_3CR1^{high} subset shows anti-inflammatory properties [58] and prevents the translocation of bacteria by phagocytosis [59]. This macrophage subset was markedly increased in NLC-D3-treated mice compared with vehicle-treated mice (Fig. 5E). Thus, the increase in the local $1,25(OH)_2D_3$ concentration by NLC-D3 may suppress the recruitment of inflammatory PMN cells and reciprocally augment anti-inflammatory CX_3CR1^{high} macrophages.

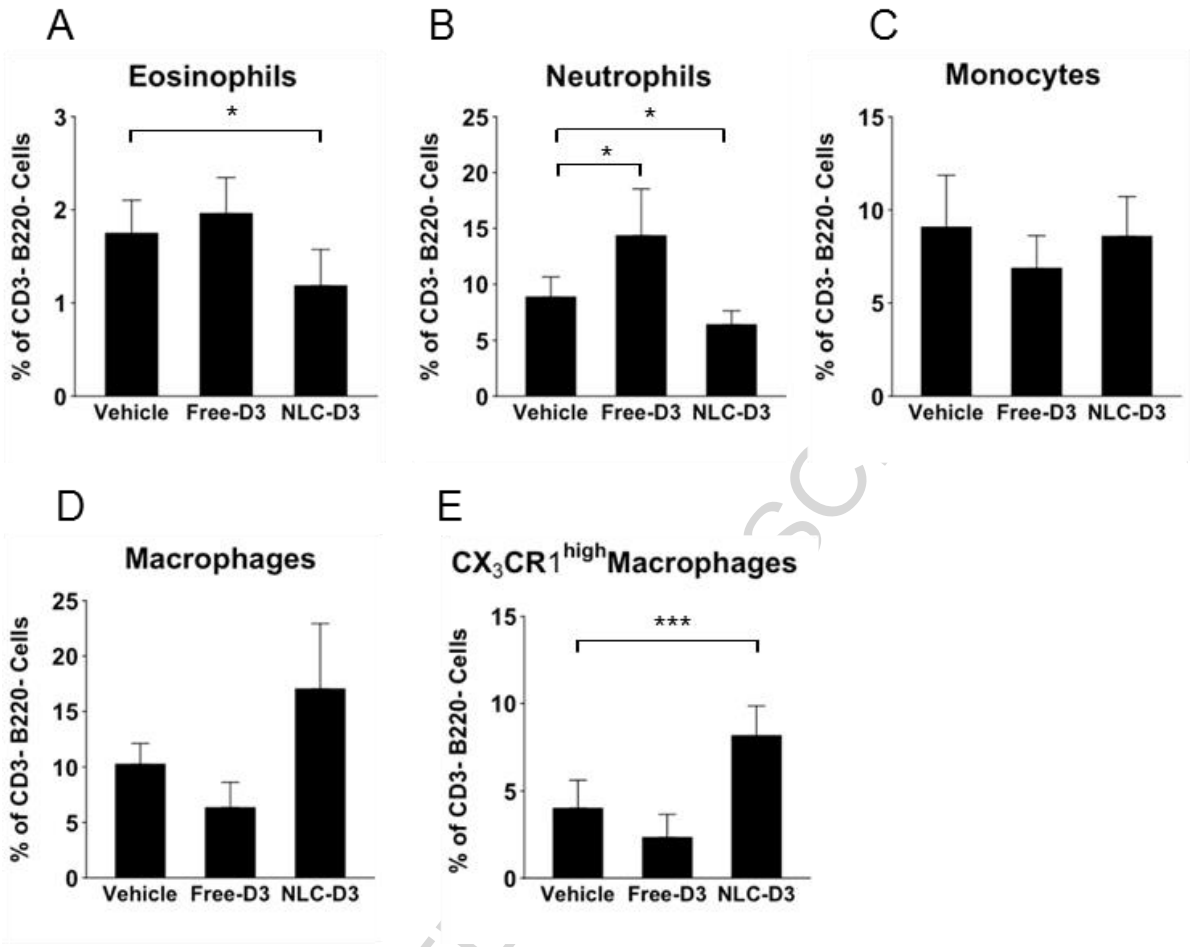

Fig. 5. Influence of NLC-D3 on the composition of immune cell subsets in the colonic lamina propria. (A–F) Colonic lamina propria cells of the mice treated with vehicle, free-D3 or NLC-D3 were analyzed 7 days after the beginning of DSS administration. Each immune cell subset was characterized based on the surface expression of specific markers. The frequency of eosinophils (A), neutrophils (B), monocytes (C), total macrophages (D), and CX_3CR1^{high} macrophages (E) in the $CD3^-B220^-$ cell gate are shown. Gating procedure and surface makers are summarized in Fig. S4 and Table S3, respectively. Values are the mean ± SD (n = 6). $^{*}P < 0.05$, $^{***}P < 0.001$ vs vehicle control. Data are representative of two independent experiments.

4. Conclusions

Herein we established procedures for the efficient incorporation of intact $1,25(OH)_2D_3$ into a NLC by employing organic solvents for emulsification at ambient temperature and including anti-oxidative components (α-tocopherol, ascorbic acid) to protect $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ in colonic tissue for at least 12 h. The continuous release of $1,25(OH)_2D_3$ from NLC-D3 enabled significant suppression of the progression of DSS colitis using a small dosage of $1,25(OH)_2D_3$. NLC-D3 suppressed the infiltration of eosinophils and neutrophils but conversely raised the population of CX_3CR1^{high} anti-inflammatory macrophages. Despite the simple formulation, NLC-D3 provided the desired therapeutic effect in an IBD model by oral administration. 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.

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