



Anti-allergic effect of inotodiol, a lanostane triterpenoid from Chaga mushroom, via selective inhibition of mast cell function

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ABSTRACT

Inotodiol is a lanostane triterpenoid found only in Chaga mushroom. In the previous study investigating anti-allergic effects of fractionated Chaga mushroom extracts, we have found evidence that purified inotodiol holds an activity to suppress the mast cell function in vivo. To address the therapeutic relevance of the finding, in this study, we investigated whether inotodiol could also alleviate allergy symptoms observed in a chicken ovalbumin (cOVA)-induced mouse model of food allergy. Like the crude 70% ethanol extract of Chaga mushroom (320 mg/kg), oral administration of inotodiol (20 mg/kg), regardless of whether that was for preventive or treatment purpose, resulted in a significant improvement in allergic symptoms and inflammatory lesions in the small intestine appearing after repeated oral challenge with cOVA. Despite the results that inotodiol (20 mg/kg) and the Chaga mushroom extract (320 mg/kg) took effect to a similar extent, immunological mechanisms underlying those effects were found to be distinct from each other. That is, the results obtained from several in vivo assays, including mast cell-mediated passive systemic anaphylaxis, activation/proliferation of adoptively transferred antigen-specific T cells and immunoglobulin (IgG1, IgE, IgA) production by antigen-specific B cells, illustrated that inotodiol selectively inhibited the mast cell function without having any noticeable effect on other immune responses while the crude Chaga mushroom extract indiscriminately suppressed diverse immune responses. The strong anti-allergic activity of inotodiol, along with its remarkable selectivity to mast cell, makes it an excellent therapeutic candidate for food allergy with both high efficacy and outstanding safety.

1. Introduction

Food allergy is an immunopathologic condition caused by an ingested food protein. While both IgE-dependent and -independent forms of food allergy have been identified, more common is the former in which mast cell also takes a central part [1–3]. When IgE located on the surface of mast cell via interaction with the high-affinity IgE receptor (IgεR) encounters a cognate food antigen in the gastrointestinal tract, a series of intracellular signaling events are triggered inside the cell. As a result, the granules containing a variety of effector molecules (e.g., histamine, neutral proteases, cytokines and heparin) are released from the cell causing allergy symptoms [4]. It is also generally accepted that allergen-specific CD4⁺ T cells, particularly Th2 CD4⁺ effector/memory T cells, play an important role in onset of food allergy. Th2 cytokines (e.g., IL-4, IL-5 and IL-13) are critical for the production of high-affinity IgE by B cells and recruitment of mast cell and eosinophil to the

afflicted tissue [5].

Inotodiol (22-hydroxy lanosterol) is a fungal secondary metabolite with a lanostane-type triterpene structure (Fig. 1A) [6]. It is found uniquely in Chaga mushroom that is a parasitic fungus to grow on birch in the forests of Northern European countries, and that has been used for centuries as a folk medicine for treatments of various pathologic conditions [7–9]. Pharmacological activities of inotodiol have been investigated in a few studies by others with a modest anti-cancer activity against several cancer cell lines being reported [10–12]. An animal study regarding the anti-cancer activity of inotodiol in a specific diabetic condition is of note [13]. Still, reports on the activity of inotodiol relevant to an immune disorder are not available up to now.

We have shown, in the previous study, that the crude 70% ethanol extract of Chaga mushroom extract (CCME) and its dichloromethane (CH₂Cl₂) fraction possess a strong anti-allergic activity [14]. It has been also shown in the same study that purified inotodiol has an activity to

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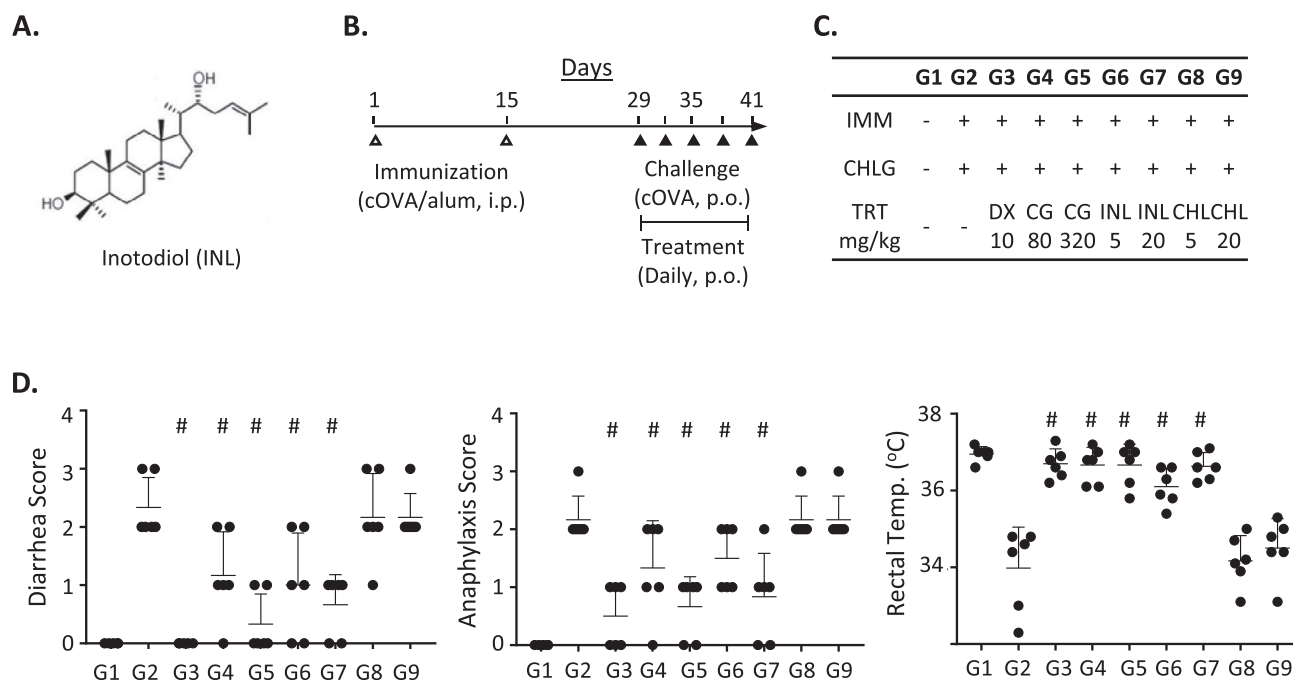


Fig. 1. Preventive effect of inotodiol on the onset of food allergy. Chemical structure of inotodiol is shown (A). The protocol for the mouse model of cOVA-induced food allergy used in this study is depicted (B). Specifics of respective experimental groups are shown in the table. The abbreviations represent immunization (IMM), challenge (CHLG), treatment (TRT), Dexamethasone (DX), Chaga mushroom extract (CG), inotodiol (INL) and cholesterol (CHL), respectively (C). The allergic symptoms, diarrhea (D left) and the behavioral abnormality due to anaphylaxis (D center), were monitored regularly (once every 30 min) for 90 min after the final challenge. The severities of diarrhea and anaphylaxis were rated from 0 to 4 according to the rating guidelines delineated in the Methods. The body temperature of mice was measured once every 30 min for 60 min after the final challenge, and the results obtained after 30 min are shown (D right). The experiment was performed with 6 mice per group. # $p < 0.01$, ANOVA.

compromise the mast cell function *in vivo*, suggesting that the anti-allergic effects of CCME and the CH_2Cl_2 fraction be at least in part attributed to the activity of inotodiol. To substantiate that notion, we explored anti-allergic effect of inotodiol with a mouse model of cOVA-induced experimental food allergy.

2. Methods

2.1. Chemicals and other reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin-glutamine solution, and dinitrophenol (DNP)-bovine serum albumin (BSA) were purchased from Thermo Fisher Scientific (Carlsbad, USA). cOVA (grade VI), alum [$\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$], eosin, hematoxylin and BSA were purchased from Sigma-Aldrich (St. Louis, USA). Toluidine blue was purchased from Samchun Chemical (Pyungtack, Korea). IL-5, IL-13 and IL-17 ELISA kits were purchased from BioLegend (San Diego, USA). Purified anti-mIgE mAb (RME-1) and biotinylated anti-mIgG1 (RMG1-1), mIgA (RMA-1) and mIgE (R36-118) were purchased BD Biosciences (San Diego, USA). Horse radish peroxidase (HRP)-conjugated streptavidin and allophycocyanin- labeled anti-CD117 (c-kit: clone 2B8) mAb were purchased from BioLegend. Anti-mouse mast cell protease-1 (MCPT-1), APC labeled anti-DO11.10 TCR (KJ-126), PE-labeled anti-CD4 (GK1.5) mAbs were purchased from eBioscience (San Diego, USA). Anti-DNP IgE was purchased from Sigma-Aldrich (St. Louis, USA). Periodic acid -Schiff (PAS) and histamine ELISA kit was purchased from Abcam (Cambridge, UK).

2.2. Mice

BALB/C mice were purchased from SAMTAKO Bio Korea (Osan, Korea). DO11.10 TCR Tg mice were purchased from Jackson Laboratory (Bar Harbor, ME), and bred in The Core Animal Facility in

Chungnam National University (CNU). Mice were maintained under a specific pathogen-free condition. All animal experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of CNU.

2.3. Preparation of CCME and inotodiol

CCME and inotodiol were prepared as described previously. Briefly, Russian Chaga mushroom (*Inonotus obliquus*) (2 kg) purchased from Kyungdong Herb Market (Seoul, South Korea) was ground into a fine powder, and extracted with ethanol (70%) in an ultrasonic bath (3 times for 2 hrs each). The crude chaga mushroom extract was then dried to a powder (300 g) after removal of the solvent under reduced pressure. This dried crude chaga mushroom extract is called CCME, was re-suspended in water and partitioned with CH_2Cl_2 to obtain the CH_2Cl_2 fraction. The CH_2Cl_2 fraction was separated by silica gel column chromatography (CC) eluting with n-hexane-acetone (100:1, 50:1, 25:1, 10:1 and 5:1) to afford five subfractions (C-1 to C-5). Subfraction C-4 (5 g) was separated again by silica gel CC eluting n-hexane-acetone (10:1) followed by further purification with RP-18 MPLC (medium pressure column chromatography) using mobile phase of $\text{MeOH-H}_2\text{O}$ (2:1, v/v). After purification by MPLC, about 250 mg of inotodiol (~97% pure) was obtained (Supp. Fig. 1).

2.4. Experimental mouse model of food allergy

Food allergy was elicited in 5- to 6-week old male BALB/C mice with cOVA as a model allergen as described previously [15,16] (see supplementary materials). The severity of diarrhea was rated with numerical scores as follows; 0-normal stool, 1-watery but morphous stool, 2-watery and amorphous stool, 3-bloody stool, 4-death. The severity of anaphylaxis (behavioral abnormality) is also rated with numerical scores as follows; 0-normal, 1-slow response on prodding, 2-no response

on prodding, 3-convulsive behavior, 4-death.

2.5. Histology

The small intestine (jejunum) was harvested 1 day after the last cOVA challenge, fixed immediately with 10% formalin, and embedded in paraffin. Thin sections (5 μ m thick) were prepared from the paraffin-embedded tissue samples in a microtome (Leica Biosystem, Germany) and stained with hematoxylin and eosin (H&E), toluidine blue and periodic acid-Schiff (PAS), respectively [17,18].

2.6. Passive systemic anaphylaxis (PSA) assay

BALB/C mice were injected i.v. with anti-DNP mIgE (5 μ g). One day after the mAb injection, the mice were injected i.v. with DNP-BSA (80 μ g). Changes in the body temperature of the mice were measured using a rectal probe thermometer once in every 50 min. for 1 hr. One hr after DNP-BSA injection, the blood was drawn from the tail vein, and the concentration of MCPT-1 in the blood was measured with ELISA [19].

2.7. β -hexosaminidase and histamine assays

Peritoneal cells were carefully collected from BALB/C mice (8 week-old) by lavage with ice cold tyrode B buffer (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO_3 , 2.7 mM KCl, 0.3 mM NaH_2PO_4 , 0.1% gelatin), pH 7.4 and washed and resuspended in tyrode A buffer (130 mM NaCl, 10 mM HEPES, 1 mM MgCl_2 , 5 mM KCl, 1.4 mM CaCl_2 , 5.6 mM glucose, 1 mg/ml BSA), pH 7.4. Then, 100 mL of the cells (5×10^5) were then loaded in a 96 well plate and cultured with either 0.5 μ g/mL of anti-mIgG or anti-mIgE mAb for 30 min. After culture, the activities of β -hexosaminidase and the concentrations histamine in the culture supernatant and the cells extracts were measured with 6.7 mM PNAG in a citrate buffer (0.6 mM Na-citrate, pH 4.5) [20] and a histamine assay kit (Abcam), respectively. The extent of release (% release) of β -hexosaminidase and histamine was calculated with a formula as follow; % release = the amount in the culture supernatant/ (the amount in the cells before culture).

2.8. In vivo T cell proliferation assay

Cells isolated from the whole body lymph nodes (LNs) of DO11.10 TCR Tg mice were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) [21] and adoptively transferred into BALB/c mice. The host mice were then immunized with cOVA (50 μ g) plus alum (2 mg). Six days after immunization, cells were isolated from the whole body LNs, and analyzed for the extent of CFSE dilution in CD4^+ DO11.10 TCR⁺ T cells.

2.9. Other methods

Other experimental methods and details of the methods described in this section can be found in supplemental methods.

2.10. Statistical analysis

The significance of the difference among the multiple groups was determined with nonparametric one-way ANOVA followed by Dunnett's multiple comparison test as post hoc analysis [22]. Two-tailed Student *t* test was used when the significance of the difference between two specific groups was calculated. Both $p < 0.01$ and $p < 0.05$ were considered statistically significant and denoted separately. All analyses were done with GraphPad Prism software (La Jolla, CA).

3. Results

3.1. Prophylactic effect of inotodiol on food allergy in an experimental mouse model

With the purpose of determining anti-allergic effect of inotodiol (Fig. 1A), we employed a mouse model of experimental food allergy using cOVA (Fig. 1B). Expectedly, when a group of sensitized (immunized) mice were treated only with the vehicle (sham treatment) during the period of oral challenge with cOVA, every mouse in the group showed severe allergy symptoms (Fig. 1C and D). As reported in the previous study, it was also clear that the severity of allergy symptoms were markedly lessened when the mice were treated with either Dexa (DX) or CCME (CG) [14].

Inotodiol treatment also led to a significant improvement in the allergic symptoms (Fig. 1C and D). Thus, when a group of sensitized mice were treated with inotodiol at a dose of 20 mg/kg, the severity of allergy symptoms decreased to the level comparable to that observed after treatment with CCME at a dose of 320 mg/kg. The dose response of inotodiol was also evident so that while inotodiol treatment at a dose of 5 mg/kg also improved allergy symptoms significantly, the level of improvement at that dose was clearly less than that at a dose of 20 mg/kg. Here, it is worth noting that inotodiol treatment even at a dose of 20 mg/kg, different from Dexa treatment (10 mg/kg), had little effect on the number of lymphocytes in the mesenteric lymph node (mLN) and the spleen as well as on the body weights of the mice (Supp. Fig. 2). Despite the structural similarity, treatment with cholesterol resulted in no improvement in allergy symptoms.

3.2. Amelioration by inotodiol of inflammatory lesions in the small intestine

Mucosal edema (i.e., thickening of the intestinal wall particularly in the areas of serosa and muscularis externa) [23], a hallmark lesion of food allergy, was readily observed in the small intestine of the sham-treated mice (Fig. 2A). And, as reported previously, treatment of the sensitized mice with either Dexa or CCME during the period of oral challenge resulted in strong improvement in the lesion. Such improvement was also evident after treatment with inotodiol. Thus, the areas of serosa and muscularis externa of the inotodiol-treated mice showed no clear sign of edema when compared with the same areas of naïve mice. Cholesterol treatment seemed to have no effect on the lesion (Fig. 2A).

Increase in the number of goblet cells in the small intestine is another hallmark of food allergy [24]. Indeed, a dramatic increase in the number of goblet cells was observed in the small intestine of sham-treated mice, when compared with that of naïve mice (Fig. 2B). In accordance with the results described above, the numbers of goblet cells found in Dexa- and CCME-treated mice were significantly lower than those found in sham-treated mice. Inotodiol treatment also resulted in a considerable decrease in the number of goblet cells in the small intestine. In contrast, the number of the cells found in cholesterol-treated mice appeared almost same as those in the sham-treated mice (Fig. 2D).

An elevation in the number of mast cells was also observed in the small intestine of sham-treated mice (Fig. 2C). That number was, however, lowered considerably when the sensitized mice were treated with either Dexa or CCME during the period of oral challenge with cOVA. Treatment with inotodiol, but not with cholesterol, also resulted in a significant decrease in the number of mast cells dwelling in the small intestine (Fig. 2E).

3.3. Selective inhibition of the mast cell-mediated immune response by inotodiol

In an effort to elucidate the immunological mechanism underlying the anti-allergic effect of inotodiol shown above, we investigated how its treatment during oral challenge with cOVA had effects on a few

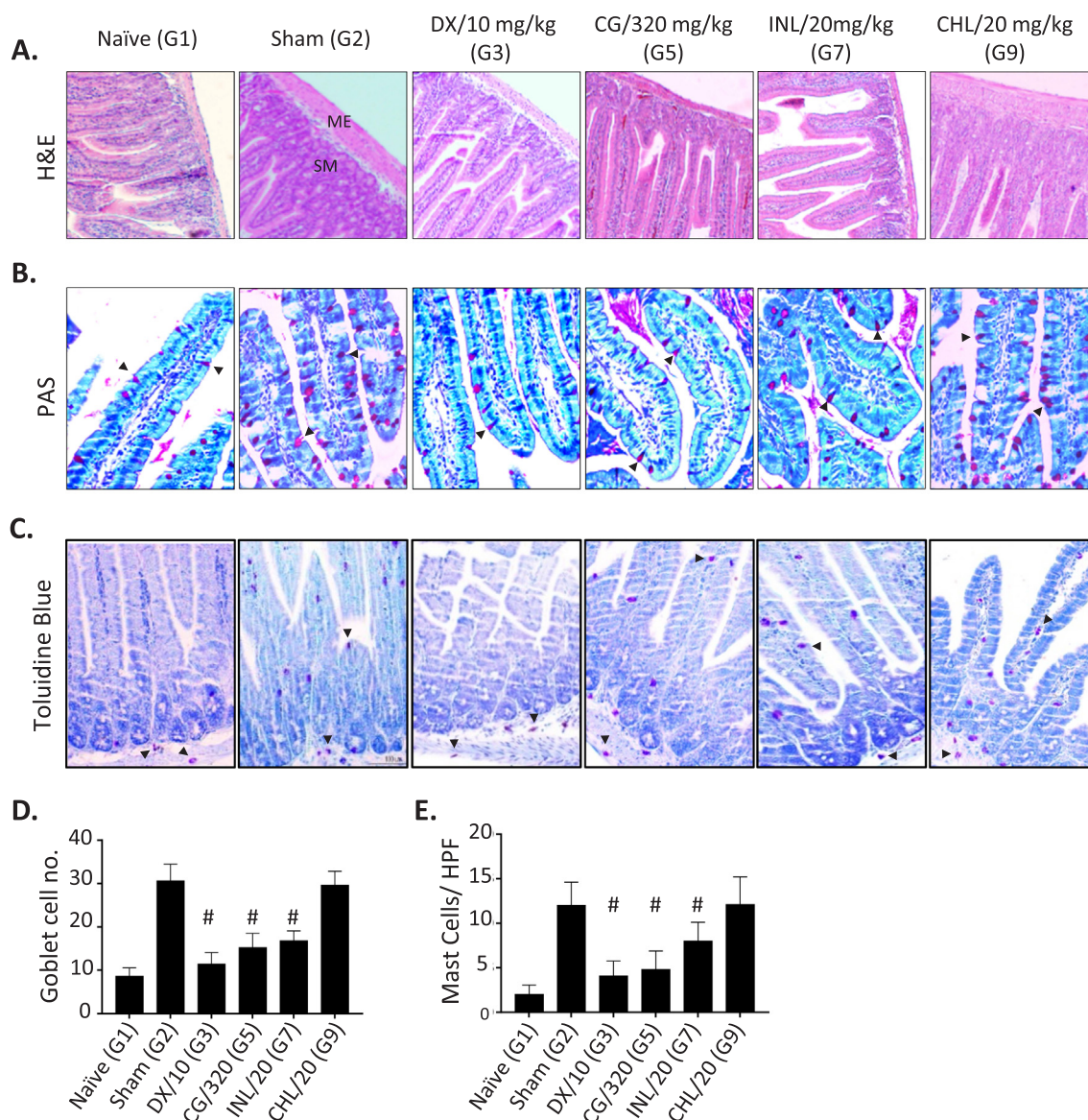


Fig. 2. Amelioration by inotodiol of the allergic inflammatory lesions in the small intestine. The small intestines (jejunum) taken from the mice one day after the final (5th) challenge (refer to Fig. 1) were cut into small pieces and fixed with formalin. Thin sections (5 mM) prepared from the paraffin-embedded tissue samples were then stained with either hematoxylin and eosin (H&E) (A), periodic acid-Schiff (PAS) (B), or toluidine blue (C) for visualization of the overall morphological changes, goblet cell, and mast cell, respectively. The areas of muscularis externa (ME) and submucosa (SM) were marked inside the picture (A). Goblet cells and mast cells were marked with arrows (B and C). The averages of the numbers of goblet cells (D) and mast cells (E) found in 10 separate high-power fields (HPFs) were plotted.

immune responses known to be deeply involved in onset of food allergy, i.e., mast cell activation/degranulation, IgE production by B cells and generation of Th2 CD4⁺ effector/memory T cells [25–27]. We first examined the effect of inotodiol on the mast cell function by analyzing the level of mast cell protease 1 (MCPT-1) in the small intestine and the blood, which is a secretory granule protease specifically expressed by mucosal mast cells (Fig. 3A). Expectedly, a high level of MCPT-1 was detected both in the small intestine and the blood from sham-treated mice whereas it was hardly detected in either specimens from naïve mice. Conforming to their strong anti-allergic effects shown above (Figs. 1 and 2), its levels in the specimens from Dexa- and CCME-treated mice were considerably lower than those from sham-treated mice. Similarly, the levels of MCPT-1 in the specimens from inotodiol-treated mice were also found diminished significantly when compared with those from sham-treated mice. Together, it was indicated that not only Dexa and CCME but also inotodiol suppressed the activation (degranulation) of mast cell triggered by cOVA administered in the course of oral challenge.

We next examined the effect of inotodiol on the production of IgE by B cells by analyzing the levels of cOVA-specific IgE in the small intestine and the blood (Fig. 3B). Expectedly, a relatively high levels of cOVA-specific IgE was detected both in the small intestine and the blood from sham-treated mice. Consistent with the results reported in the previous study [14], CCME treatment caused a dramatic decrease in the level of cOVA-specific IgE both in the small intestine and the blood while Dexa treatment had effect only on the level of IgE in the small intestine but not in the blood. Of note, different from Dexa and CCME, treatment with inotodiol resulted in no significant change in the level of cOVA-specific IgE either in the small intestine or in the blood.

We also examined the effect of inotodiol on the generation of cOVA-specific CD4⁺ effector/memory T cells by analyzing the levels of Th cytokines produced by T cells from the draining lymph node (mesenteric lymph node) during culture with cOVA in vitro (Fig. 3C). Expectedly, high levels of IL-5, IL-13, and IL-17 were detected in the culture supernatants of the cells from sham-treated mice while those levels in the supernatants of the cells from naïve mice were

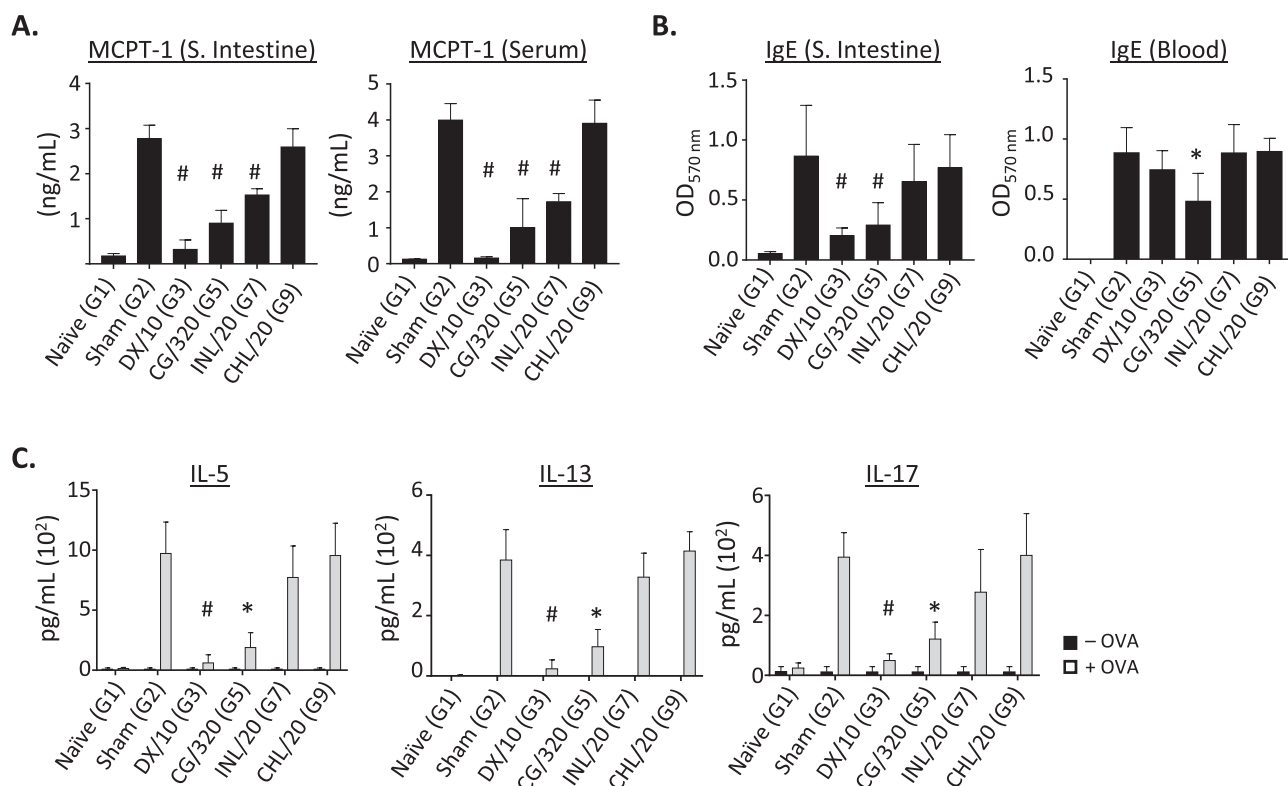


Fig. 3. Selective suppression of mast cell-mediated immune responses by inotodiol. The concentrations of MCPT-1 (A) and cOVA-specific IgE (B) in the extracts of the small intestines and the blood taken from the mice one day after the final challenge (refer to Fig. 1) were measured with ELISA. The cells were isolated from the mesenteric lymph nodes one day after the final challenge, and cultured with or without cOVA for 3 days. The concentrations of IL-5, IL-13 and IL-17 in the culture supernatant were then measured with ELISA, respectively (C). # $p < 0.01$, * $p < 0.05$, respectively, ANOVA.

unmeasurably low. Also, as reported in the previous study [14], the cytokine levels in the culture supernatants of the cells from Dexa- or CCME-treated mice were considerably low relative to those of the cells from sham-treated mice, suggesting that cOVA-specific CD4⁺ T cell immune responses against cOVA administered in the course of oral challenge was strongly suppressed by Dexa and CCME. In striking contrast to the results obtained with the cells from Dexa- or CCME-treated mice, however, the levels of those cytokines in the culture supernatants of the cells from inotodiol-treated mice were not significantly different from those of the cells from sham-treated mice.

3.4. Amelioration of IgE-mediated passive anaphylaxis by inotodiol

Even though the result shown above for MCPT-1 suggested suppression of the mast cell function by inotodiol (Fig. 3A), it was also conceivable that the decrease in MCPT-1 in the small intestine and the blood following the inotodiol treatment was mainly due to the decrease in the number of mast cells in the small intestine. In order to confirm suppression of the mast cell function in vivo by inotodiol, we employed a mouse model of IgE-mediated passive systemic anaphylaxis. Note that decrease in the body temperature and increase in the concentration of MCPT-1 in the blood observed after i.v. injection of DNP-BSA (the model allergen used) were used as readouts for the levels of anaphylactic reactions caused by mast cells (Fig. 4A), and that anti-anaphylactic effect of inotodiol at 50 mg/kg was no less than those of CCME at 320 mg/kg and Dexa at 10 mg/kg, respectively.

Dexa, CCME and inotodiol commonly improved the anaphylactic symptoms in a dose-dependent manner (Fig. 4A). Even though treatment with Dexa at 10 mg/kg seemed to yield the biggest improvement, it has to be noted that the same treatment also had an adverse effect on the viability of various immune cells including mast cells (Supp. Fig. 3 and see below). Treatments with CCME and inotodiol at 320 mg/kg and

50 mg/kg doses, respectively, resulted in an improvement in the anaphylactic symptoms to an extent comparable to that with Dexa at 10 mg/kg. Yet, different from Dexa, CCME and inotodiol seemed to have no effect on the viability of immune cells including mast cells.

We also examined duration of treatment required for inotodiol to take effect by varying the number of its treatments (once a day) (Fig. 4B). When mice were treated only one time, just 30 min before administration of DNP-BSA, little improvement in the anaphylactic symptoms was observed. A significant improvement began to be observed after two days of treatment, and the level of improvement increased further when mice were treated three days in a row. The difference in the degrees of improvement observed after three and four days of treatments was only marginal.

3.5. Diminution of mast cell function by inotodiol

The peritoneal cavity is an excellent source of mast cell; mast cells represent almost 2% of total peritoneal exudate cells (PECs). In order to confirm more directly whether inotodiol attenuated the immunoreactivity of mast cells in vivo, we isolated PECs from sham- and inotodiol- and other drug-treated mice, respectively, and stimulated mast cells in PECs with anti-IgE mAb to induce degranulation. As noted above, prior to incubation of PECs with the mAb, we carefully enumerated the numbers of PECs collected from the peritoneal cavities, and also analyzed the percentage of mast cells (c-kit⁺/IgE⁺ double positive cells) in PECs with flow cytometry. Any of the reagents used for treatment, except Dexa, had no measurable effects either on the number of PECs or on the percent of mast cells (Supp. Fig. 3).

We next incubated PECs with anti-IgE mAb and measured the amounts of mast cell granule-specific molecules (i.e., β -hexosaminidase and histamine) released to the supernatants [20]. Note that the same numbers of PECs were used in the assays. Expectedly, significant levels

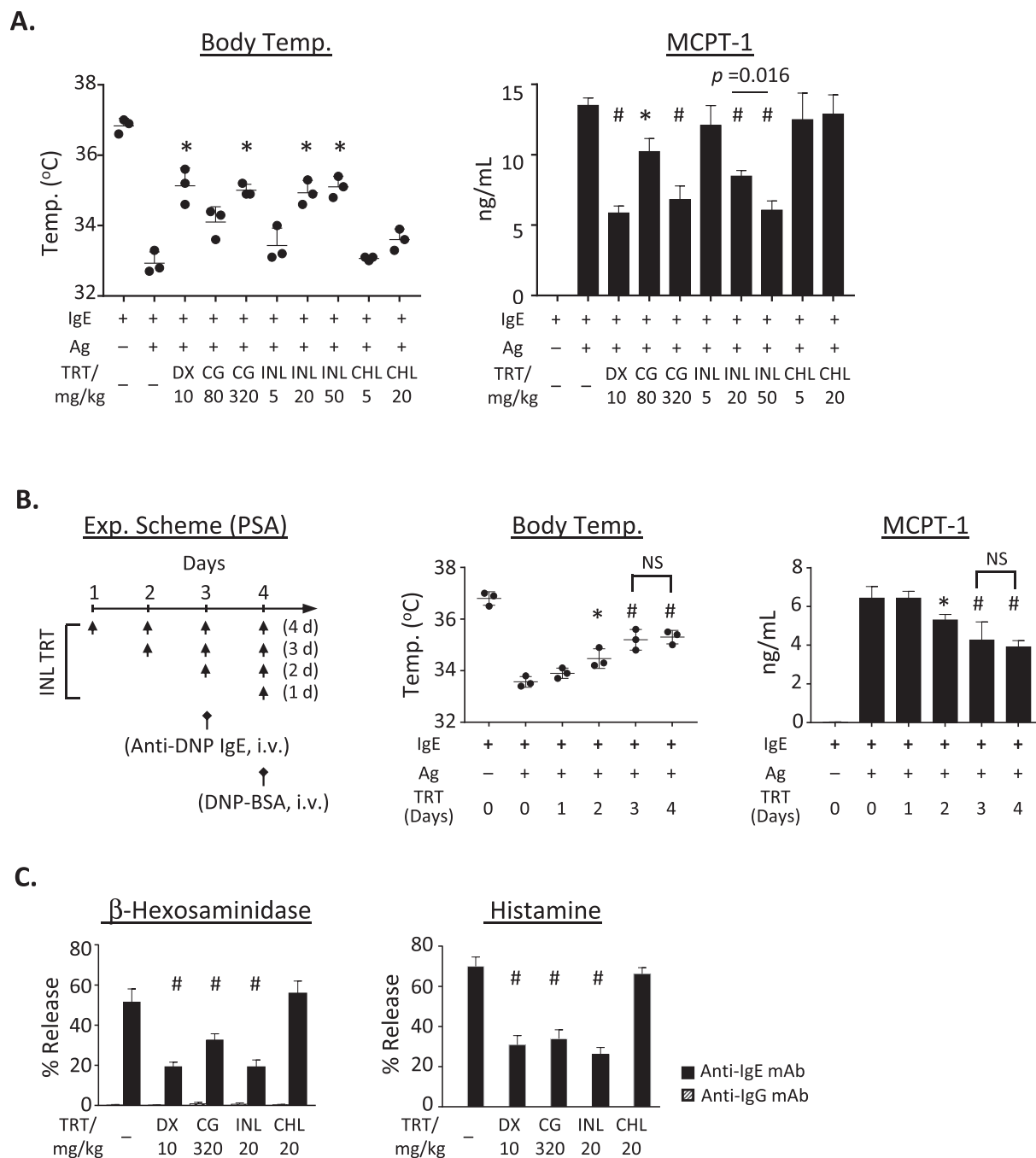


Fig. 4. Amelioration by inotodiol of IgE-mediated passive systemic anaphylaxis via suppression of mast cell function. Mice were treated with vehicle alone (-) or with the indicated doses of dexamethasone (DX), crude Chaga mushroom extract (CG), inotodiol (INL), and cholesterol (CHL), respectively, for 4 days. Anti-DNP-IgE and DNP-BSA were injected i.v after 3 and 4 days of treatments, respectively. Thirty minutes after injection of DNP-BSA, the body temperature was measured (A, left). The concentrations of MCPT-1 in the blood taken one hour after injection of DNP-BSA were measured with ELISA (A, right). The duration of inotodiol treatment (20 mg/kg) were varied from 1 to 4 days (once a day) before injection of DNP-BSA as depicted (B, left). The body temperature and the concentration of MCPT-1 were measured as described in A. Total peritoneal cells were collected from the mice treated with vehicle alone (-) or with the indicated doses of respective reagents for 4 days, and cultured for 30 min with either anti-mIgG (hatched bar) or anti-mIgE mAb (solid bar). The levels of β-hexosaminidase (C, left) and histamine (C, right) released to the supernatants and in the cells were measured separately to calculate the levels of degranulation. The experiments were performed with 3 mice per group. # $p < 0.01$ and * $p < 0.05$, respectively, ANOVA.

of β-hexosaminidase and histamine were detected in the supernatants when PECs from sham-treated mice were incubated with anti-IgE mAb but not with anti-IgG mAb (Fig. 4C). Conforming to the results from the experiments for PSA (Fig. 4A), the amounts of β-hexosaminidase and histamine detected in the supernatants of PECs from Dexa- or CCME-treated mice were found to be considerably lower than those from sham-treated mice. Similar results were obtained with PECs from

inotodiol-treated mice; thus, the levels of β-hexosaminidase and histamine released to the supernatants by PECs following incubation with anti-IgE mAb also decreased profoundly.

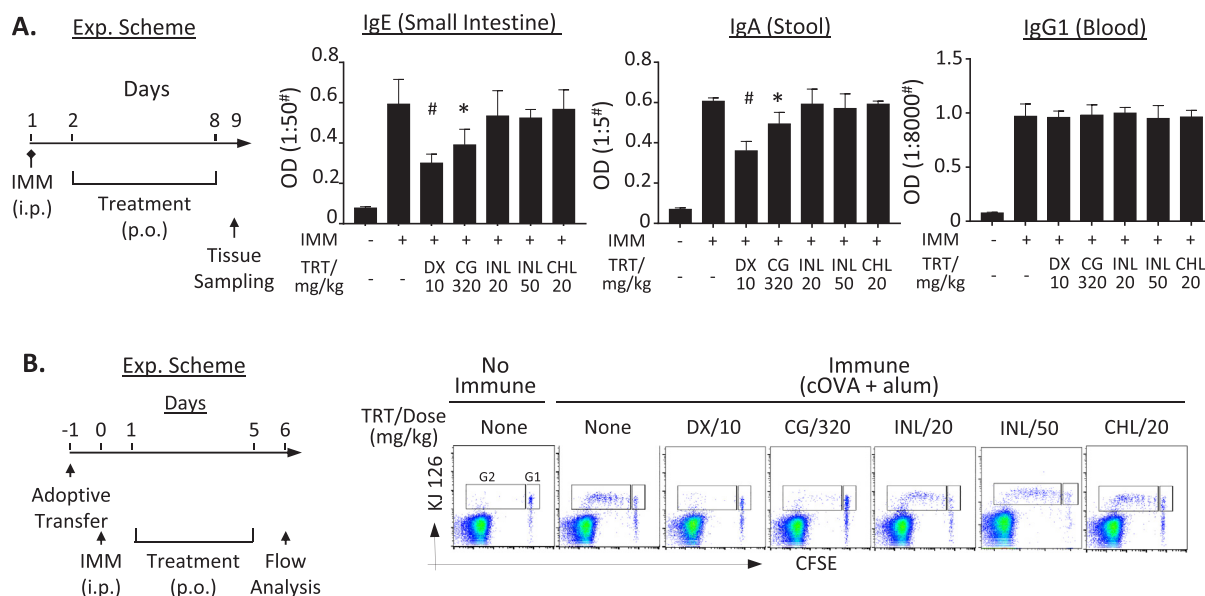


Fig. 5. Lack of inhibition of antigen-specific B and T cell immune responses by inotodiol. Mice were immunized i.p. with cOVA plus alum and treated for 7 days with vehicle alone (-) or with the indicated dose of dexamethasone (DX), Chaga mushroom extract (CG), inotodiol (INL), and cholesterol (CHL), respectively (A, left). One day after the last treatment, the small intestine extracts, stool extracts and blood serum were prepared and the levels of cOVA-specific IgE, IgA, and IgG1 were measured with ELISA, and plotted (A). Cells were isolated from the whole-body lymph nodes (LNs) of DO11.10 TCR Tg mouse, and labeled with CFSE and adoptively transferred to BALB/c mice. The host mice were, then, immunized i.p. with cOVA plus alum, and treated for 5 days with vehicle alone (-) or with the indicated dose of respective reagents. One day after the last treatment, cells were isolated from the whole-body LNs of the host mice, and labeled with anti-CD4 plus anti-DO11.10 TCR (KJ 126) mAbs, and analyzed for the extents of CFSE dilution in adoptively transferred DO11.10 CD4⁺ (KJ 126⁺) T cells. The gated CD4⁺ T cells are shown (B). The ratio between non-proliferating (G1) and proliferating (G2) DO11.10 CD4⁺ T cells were also plotted (Supp. Fig. 3). # $p < 0.01$ and * $p < 0.05$, respectively, ANOVA.

3.6. Lack of inhibition by inotodiol of antigen-specific B cell and T cell immune responses

The results shown above (Fig. 3B and C) suggested that the effects of inotodiol on cOVA-specific B and T cell immune responses were quite minimal, if any. In order to ascertain the effects of inotodiol on antigen-specific B and T cell immune responses in vivo, we carried out separate experiments. We first examined its effect on an antigen-specific B cell immune response by immunizing mice with cOVA plus alum and analyzing the levels of cOVA-specific IgE, IgA and IgG1 in the small intestine, the stool and the blood, respectively, eight days after immunization (Fig. 5A). Expectedly, relatively high levels of cOVA-specific IgE and IgA were detected in the specimens from sham-treated mice, and, also not surprisingly, those levels decreased significantly when mice were treated with either Dexa (10 mg/kg) or CCME (320 mg/kg). In contrast, treatment with inotodiol appeared to have little effect on the immune response; thus, the levels of cOVA-specific IgE and IgA in the specimens from the mice treated with inotodiol even at a dose of 50 mg/kg were not measurably different from those in the specimens from sham-treated mice. In contrast to IgE and IgA, the levels of IgG1 in the blood did not change noticeably after treatments with any of those reagents (Fig. 5A).

We next examined an effect of inotodiol on antigen-specific T cell immune responses by analyzing the extent of proliferation (i.e., CFSE dilution) of adoptively transferred cOVA-specific DO11.10 CD4⁺ TCR Tg T cells following immunization of host mice with cOVA plus alum (Fig. 5B, Supp. Fig. 4). Expectedly, it was clear that a proliferation of DO11.10 TCR Tg T cells proceeded vigorously when the host mice were treated with the vehicle alone (sham treatment) after immunization, and also that the extent DO11.10 T cell proliferation greatly reduced when they were treated with either Dexa or CCME. Surprisingly, however, the extent of T cell proliferation observed in inotodiol-treated mice appeared not much different from that in sham-treated mice, indicating that inotodiol had very little, if any, effect on the proliferation

of DO11.10 CD4⁺ T cells induced by cOVA plus alum as an adjuvant.

3.7. Prevention by inotodiol of the recurrence of food allergy in an experimental animal model

Even though we had shown above that the treatment with inotodiol prevented onset of food allergy in the mouse model (refer to Fig. 1), it was not known whether the same treatment could prevent recurrence of food allergy symptoms in mice that had already developed the allergy. To address the issue, we treated the mice that had been repeatedly challenged (5 times) with cOVA beforehand with inotodiol, and re-challenged them with cOVA to cause recurrence of food allergy (Fig. 6A and B).

As reported in the previous study, treatment of allergic mice with Dexa (10 mg/kg) or CCME (320 mg/kg) ameliorated the allergy symptoms shown after re-challenge with cOVA (Fig. 6C). Of note, treatment of the mice with inotodiol (50 mg/kg) also improved the allergy symptom to a significant extent so that none of the mice showed severe allergy symptoms like bloody stool, convulsive behavior, and dramatic drop in the body temperature. Different from the results shown in Fig. 1 for the prophylactic effects, however, the treatment effects of CCME and inotodiol were seen only at the high doses; i.e., 320 mg/kg of CCME and 20 mg/kg of inotodiol. Consistent with amelioration of the allergy symptoms, the inflammatory lesions in the small intestine (edema and increase in the number of goblet cells) were also improved markedly after treatment with inotodiol (Fig. 6D and E). The level of MCPT-1 in the blood of the inotodiol-treated mice was also found to be lowered significantly relative to that of sham-treated mice (Fig. 6F).

4. Discussions

We report here for the first time that inotodiol holds a unique immuno-regulatory activity to lead to amelioration of allergy symptoms in

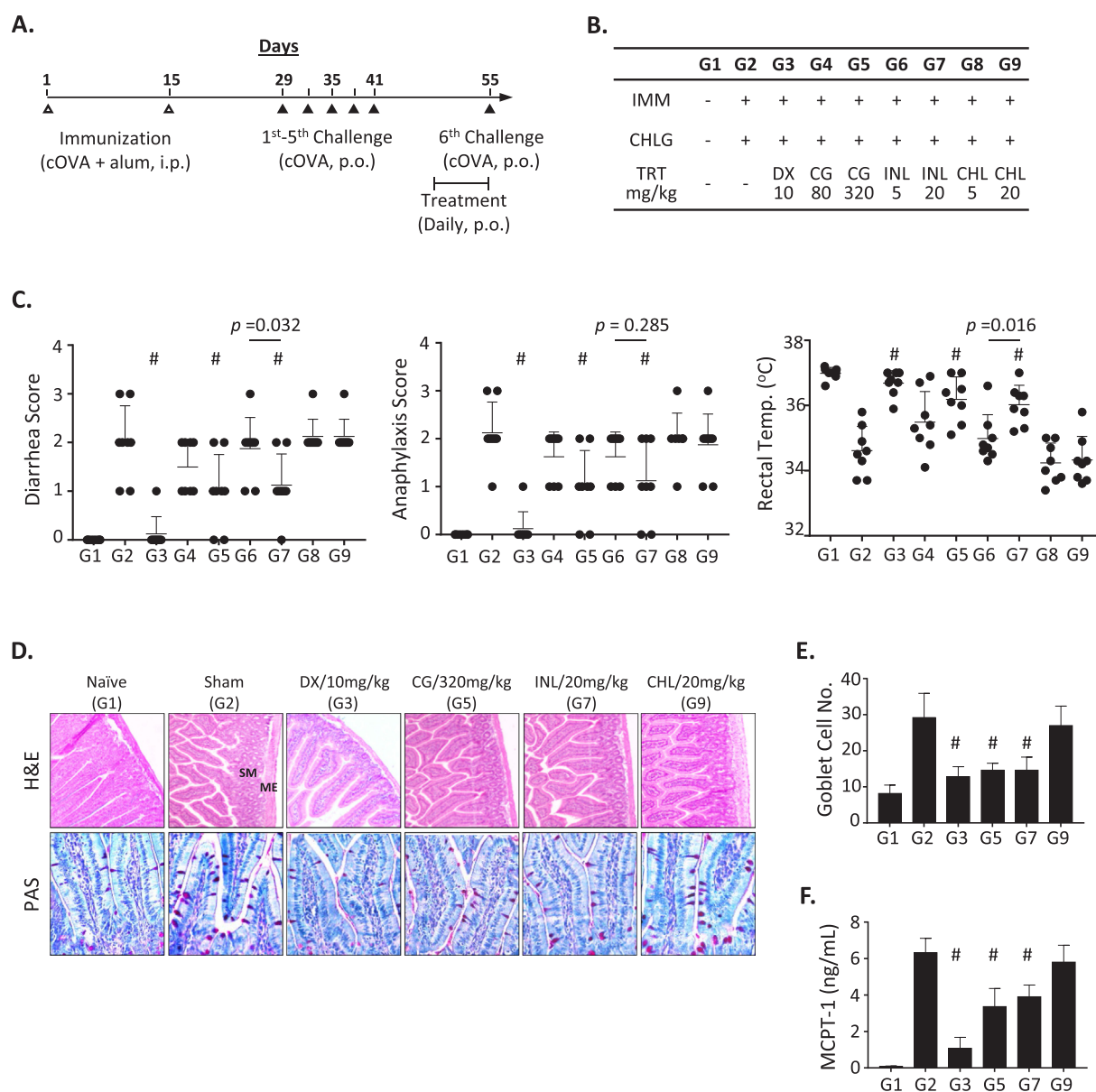


Fig. 6. Therapeutic effect of inotodiol on recurrence of food allergy. BALB/C mice were immunized with cOVA plus alum twice and challenged with cOVA 5 times. Seven days after the 5th challenge, the allergic mice were treated daily with the vehicle alone (-) or with dexamethasone (DX), Chaga mushroom extract (CG), inotodiol (INL) and cholesterol (CHL), respectively, for 7 days. Shortly after the last treatment, they were challenged with cOVA to elicit the allergic reaction (A and B). Allergy symptoms, i.e., diarrhea (C, left) and the behavioral abnormality due to anaphylaxis (C, center), were monitored regularly (once every 30 min) for 90 min after the final (6th) challenge. The severities of diarrhea and anaphylaxis were rated from 0 to 4 based on the rating guidelines noted in the Methods. The body temperature was measured once every 30 min for 60 min after the final challenge, and the results obtained after 30 min are shown (C, right). The small intestines (jejunum) taken from the mice one day after the final challenge were cut into small pieces and fixed with formalin. Thin sections (5 μ m) prepared from the paraffin-embedded tissue samples were then stained with either hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) for visualization of the overall morphology (D, top) and goblet cell (D, bottom), respectively. The areas of muscularis externa (ME) and submucosa (SM) were marked inside the picture. The averages of the numbers of goblet cells found in 10 separate high-power fields were plotted (E). The concentrations of MCPT-1 in the blood taken from the mice one day after the final challenge were measured with ELISA (F). The experiment was performed with 8 mice per group. # $p < 0.01$, ANOVA.

a mouse model of food allergy. Even though CCME also resulted in robust improvement of food allergy symptoms, the impact of CCME and inotodiol on overall immune landscape appeared quite different. Results from this study clearly indicate that inotodiol acts predominantly on mast cell to compromise its immunological function while CCME has effect on a variety of immune responses induced by orally administered cOVA. Given the fact that CCME is composed of a number of different molecules with distinct chemical structures and biological activities, modulation by CCME of various types of immune responses is not surprising [28–30]. In that regard, diverse biological activities of betulinic acid, a pentacyclic triterpenoid abundant in

Chaga mushroom, is worth noting [31]. Like other pentacyclic triterpenoids (e.g., oleanolic acid and ursolic acid, etc.), betulinic acid has been studied extensively and various immuno-modulatory activities have been identified [32,33].

The result that it took at least three days for inotodiol to take full effect in IgE-mediated passive systemic anaphylaxis model indicates that inotodiol is a slow acting mast cell stabilizer [34]. Even though the reason for delay in action of inotodiol is not yet clear, one may envision a couple of different scenarios. Firstly, it is possible that inotodiol interferes with development/differentiation of mast cells from their precursor cells, and thus that the number of mast cells decreases

progressively during the treatment [35]. Secondly, it is also possible that it takes time for mast cell to undergo phenotypic change by inotodiol. For example, inotodiol might interact with co-repressor of a transcription factor to alter the levels of key proteins involved in degranulation and/or FcεRI signaling [36]. In this case, it may take longer for inotodiol to lower the levels of those proteins below the threshold levels. We think the first scenarios is unlikely as the number of mast cells in the peritoneal cavity did not change at all even after four days of inotodiol treatment. The second scenario is still plausible, even though no direct evidence to support that scenario is available yet.

Mechanism of action (MOAs) of inotodiol for the selective inhibition of mast cell function is so far not clear. Yet, considering that inotodiol is an oxysterol derived directly from lanosterol (a key common intermediate of ergosterol, cholesterol and phytosterol biosynthesis) via hydroxylation at carbon 22 (C-22) [37], the studies by others regarding the immunoregulatory activities of several sterols and oxysterols are worth noting [38,39]. According to those studies, immunoregulatory activities of specific sterols and oxysterols can be divided broadly into two separate categories. Firstly, several sterols and oxysterols represented by desmosterol and 27-hydroxy Cholesterol (27-OHC), respectively, act as ligands for regulators (e.g., SCAP, INSIG) of specific transcription factors such as LXR (liver X receptor) and SREBP (sterol regulatory element-binding protein) [38]. Consequently, they are deeply involved in regulation of the transcription of various genes in immune cells. Secondly, oxysterols also function as ligands for a receptor expressed on the surface of immune cells. According to the studies by Hanedouche et al. [40] and Liu et al., [41] 7α, 25-OH cholesterol (7α, 25-OHC) interacts with EBI2 (Epstein-Barr virus-induced G-protein coupled receptor 2) expressed on the surface of B cells at a subnanomolar concentration. As a result, B cells fail to migrate to appropriate follicular sites in the secondary lymphoid organs, and cannot receive T cell help for antibody production and differentiation into plasma cells. We speculate that the MOA of inotodiol may mimic one of those mechanisms noted above. Studies are currently underway to understand how inotodiol works on mast cell to compromise their immune function.

The results that inotodiol selectively inhibits mast cell function to improve food allergy symptoms are quite encouraging for an attempt to develop a food allergy medication in use of inotodiol. It is well-known that Dexamethasone, one of the most potent and widely used allergic medications, has also multiple side-effects damaging various tissues and organs [42]. It is also highly conceivable that long-term use of Dexamethasone even at a low dose may allow patients more susceptible to infection and cancer as it suppresses not only the types of immune cells directly related with allergy but also other types of immune cells essential for defense against infection and cancer. Long-term use of CCME for prevention of food allergy may cause similar health issue as CCME also modulates multiple immune responses. In light of that, it is of special note that inotodiol, different from Dexamethasone and CCME, acts predominantly on mast cell, for it implies that inotodiol can be developed as a safer allergy medication with less unwanted effects. Those characteristics must be of particular importance when allergy patients are children and adolescences [43].

CRediT authorship contribution statement

Thi Minh Nguyet Nguyen: Investigation, Methodology, Data curation, Formal analysis. **Hong Son Le:** Investigation, Methodology, Data curation. **Ba Vinh Le:** Investigation, Methodology. **Young Ho Kim:** Supervision, Conceptualization. **Inkyu Hwang:** Supervision, Conceptualization, Data curation, Funding acquisition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2020.106244>.

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Materials and Methods

Preparation of saline-tween 20 emulsion of a testing reagent for oral gavage

A pulverized testing reagent was thoroughly mixed with tween 20 in a ceramic dish, and the resulting mixture was emulsified with saline (a volume 99 times than that of tween 20)¹. The testing reagent-tween 20-saline emulsion (0.2 mL) was then administered directly to the stomach of mice.

Experimental mouse model of cOVA-induced food allergy

BALB/C (5- to 6-week old, male) were immunized i.p. with cOVA (20 µg) plus alum (2 mg) twice (once every two weeks)^{2,3}. One week after the second immunization, the blood was drawn from the immunized mice, and the level of cOVA-specific IgE in the blood was measured with ELISA. The mice producing cOVA-specific IgE at a concentration in the range of mean $\pm$ SD were chosen and randomly divided into seven groups (6 mice per group). Two weeks after the second immunization, the mice were challenged p.o. with cOVA (50 mg) five times (once every three days). During the period of the oral challenge with cOVA, the mice were treated daily p.o. with vehicle alone, Dexa, cholesterol, CCME and inotodiol, respectively. After the fifth challenge, the body temperature of the mice was measured using a rectal probe thermometer (Physitemp, Clifton, USA); once every 30 min for 60 min. Diarrhea and the behavioral abnormality due to anaphylaxis were also monitored regularly; once every 30 min for 90 min⁴.

Histology

Histopathology examinations were performed as described previously⁵. Formalin-fixed paraffin embedded tissue samples were sliced into sections of 5- μ m thickness in a microtome (Leica Biosystem, Germany). The thin sections were then stained with hematoxylin and eosin (H&E). Mast cell was stained with toluidine blue⁶. Mucosal goblet cell was stained with PAS (Abcam) according to the manufacturer's manual. Microscopic fields for examination were chosen randomly and viewed at magnifications of x100, x200, x400, respectively. A minimum of 10 areas was scored per section.

Preparation of tissue (the small intestine) extracts⁷

The small intestine was cut into 5 cm pieces one day after the final oral challenge with cOVA. The tissue fragments were washed with cold 1x PBS and snap-frozen using a dry-ice EtOH bath. The tissue sample was then transferred into a mortar containing liquid nitrogen (LN2) and ground with a pestle. The ground tissue sample was quickly transferred into a microcentrifuge tube (1.5 mL) and mixed with 500 μ L of the extraction buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1mM EDTA, 10% glycerol, 1 mM PMSE, and a cocktail of freshly prepared protease inhibitors). After incubation for 1.5 hrs at 4°C, the tissue lysate was centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was then aliquoted and stored at -70°C. The protein concentration of the tissue lysate was measured with BCA method.

Measurement of the concentrations of cOVA-specific Igs and MCPT-1 in the blood

The blood obtained from the tail vein were centrifuged at 3,000 rpm for 15 min at 4°C to obtain

the plasma. The plasma samples were stored at -80°C until further analysis. cOVA-specific Igs were measured as described previously with ELISA ⁴. The concentrations of mouse MCPT-1 in the plasma were also measured with ELISA ⁸.

Measurement of the levels of cytokine production by cOVA-specific T cells

Single cell suspensions were prepared from the mesenteric lymph nodes (mLNs) harvested 1 day after the fifth challenge with cOVA (refer to Fig. 1). Two hundred microliters of the LN cells (1×10^6 cells) in the complete RPMI medium (10% FBS, 10 mM HEPES, 50 μ M 2-mercaptoethanol, 1x Penicillin-Streptomycin) were cultured with (or without) cOVA (100 μ g/mL) for 3 days in a 37°C humidified CO₂ incubator. The culture supernatants were then collected, and the concentrations of IL-5, IL-13 and IL-17 were measured with ELISA.

Passive systemic anaphylaxis assay.

BALB/C mice treated p.o. with the vehicle alone, Dexamethasone, CCME, inotodiol, or cholesterol for 3 straight days were injected i.v. with anti-DNP mIgE (5 μ g). One day after the mAb injection, the mice were treated with the respective reagents one more time. Two hrs after the last treatment, they were injected i.v. with DNP-BSA (80 μ g)⁹. Thirty min after the injection, changes in the body temperature of the mice were measured using a rectal probe thermometer. One hr after DNP-BSA injection, the blood was drawn from the tail vein, and the concentration of MCPT-1 in the blood was measured with ELISA.

Antibody production assay

BALB/C mice were injected i.p with cOVA (50 µg) plus alum (2 mg) for immunization. Beginning 24 hrs after the immunization, the mice were treated p.o. with the vehicle alone, Dexa, CCME, inotodiol or cholesterol for 7 straight days. One day after the last treatment, the blood, the small intestine and the stool were collected to measure the levels of cOVA-specific IgG1, IgE and IgA, respectively. Briefly, the stools were frozen quickly at -80°C. The frozen fecal samples (200 mg) were thawed and homogenized in 0.5 mL of the extraction solution (1x PBS pH 7.0, 2 mM PMSF, 1 mM NaN₃). The fecal suspensions were spun at 1,500 rpm for 20 min at 5°C¹⁰. The concentration of IgA in the supernatants were measured with ELISA assay.

Measurement of the level of T cell proliferation in vivo

The whole body lymph nodes (LNs) were harvested from DO11.10 TCR Tg mice, and a single cell suspension was prepared. The lymph node cells were then labeled with CFSE as described previously^{11, 12}. The CFSE-labeled crude LN cells (1×10^7) were adoptively transferred into BALB/c mice. One day after adoptive transfer, the host mice were injected i. p. with cOVA (50 µg) plus alum (2 mg). Beginning 24 hrs after the immunization, the mice were treated p.o. with the vehicle alone, Dexa, CCME, inotodiol or cholesterol for 5 straight days. Six days after the immunization, cells were isolated from the whole body LNs of the mice, and stained with PE-labeled anti-mCD4 plus APC-labeled anti-DO11.10 TCR (KJ126) mAbs for analysis of the level

of CFSE dilution in CD4⁺DO11.10⁺ T cells. FACSCantoTM flow cytometer (BD Bioscience) was used for flow analysis.

Peritoneal mast cell degranulation assay

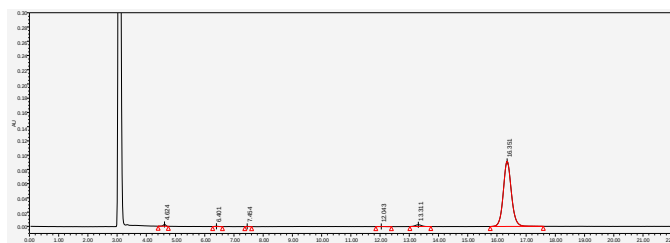
The male BALB/C mice (11 weeks old) were divided into groups and pretreated with vehicle alone or respective test reagents for 4 days. The, the mice were scarified and the peritoneal cavity was lavaged. Briefly, 6 ml of tyrode B buffer (137mM NaCl, 5.6 mM glucose, 12mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaHPO₄, 0.1 % gelatin) was injected into the peritoneal cavity and carefully recovered after massaging the abdomen gently several times. Peritoneal cells were collected by centrifugal at 1200 rpm for 5 min, 10⁰C. The cell pellet was resuspended and washed twice in tyrode B buffer. The number of peritoneal cells was counted and diluted to 5x10⁶ cells/ml in tyrode A buffer (130 mM NaCl, 10 mM HEPES [pH 7.4], 1 mM MgCl₂, 5 mM KCl, 1.4 mM CaCl₂, 5.6 mM glucose, 1 mg/ml BSA).

Two hundred microliter of the peritoneal cells was then loaded into 96 well plate and prewarmed for 30 min at 37⁰C. The plated cells were mixed with either anti-mIgE (0.5 µg/ml) or anti-mIgG (0.5µg/ml), and incubated for 30 min in 37⁰C humidified CO₂ incubator ^{13, 14}. After culture, the activities of β-hexosaminidase and the concentrations of histamine in the culture supernatants and the peritoneal cell extracts were measured separately.

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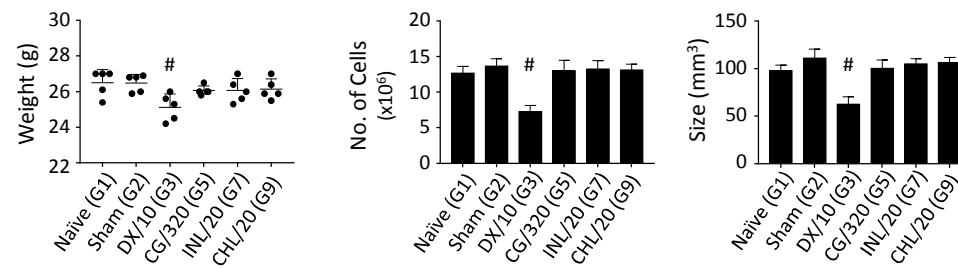
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	Retention Time	Area	% Area	Height
1	4.624	16674	0.91	2591
2	6.401	3717	0.20	402
3	7.454	1547	0.08	266
4	12.043	1786	0.10	141
5	13.311	36441	1.99	2448
6	16.351	1769940	96.71	91474

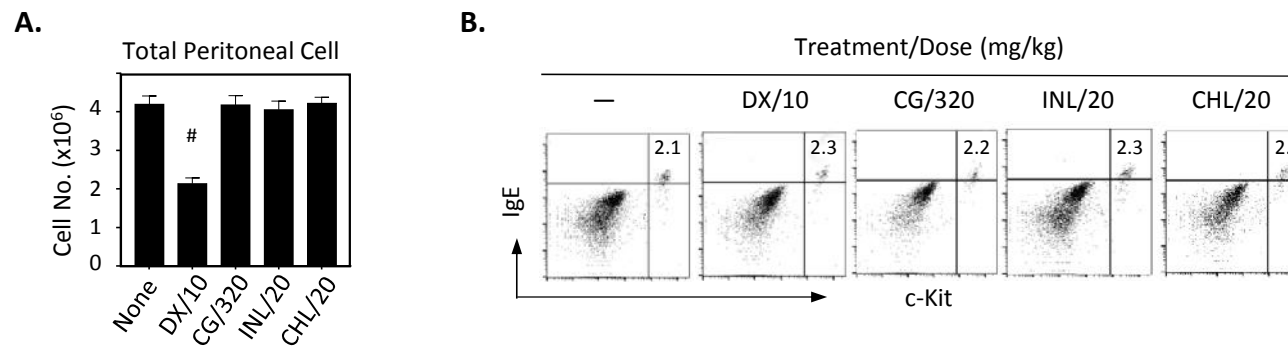
Supp. Figure 1. HPLC analysis of purified inotodiol

Purified inotodiol was subjected to HPLC analysis for determining the purity. The HPLC peak with retention time of 16.351 was identified as inotodiol by mass spectrometry and NMR spectroscopy. The calculated peak area indicated that the purity of inotodiol was > 96%.



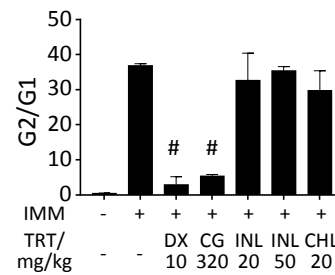
Supp. Figure 2. Inotodiol treatment causes no change in the body weight, cellularity of the LN, and size of the spleen

Shortly after the 5th challenge with cOVA (refer to Fig. 1) the body weights of the mice were measured. One day after the 5th challenge, the mice were dissected to harvest the mesenteric LNs (mLNs) and the spleens. Single cell suspensions were made from the mLNs, and the total cell numbers in the suspensions were enumerated. The lengths and the widths of the spleens were also measured to estimate the sizes (volumes) of the spleens. The treatment with CCME (CG), inotodiol (INL), or cholesterol (CHL) appeared to have no effect on any of those measures while the treatment with Dexamethasone (Dexa) caused marked changes in them. #, $p < 0.01$, ANOVA.



Supp. Figure 3. No alteration in the number of peritoneal mast cells following inotodiol treatment.

BALB/C mice were treated *p.o.* with the vehicle alone (-) or with dexamethasone (DX), crude Chaga mushroom extract (CG), inotodiol (INL) and cholesterol (CHL), respectively, for 4 days. After the treatment, peritoneal cells were harvested, and the numbers of total peritoneal cells were enumerated. Average numbers of the cells in respective groups of mice and their SDs were plotted. No significant changes in the average cell numbers were observed among the experimental groups, except the Dexamethasone-treated group (**A**). Total peritoneal cells were stained with anti-c-kit plus anti-mIgE mAbs and analyzed with flow cytometry to identify the mast cell population (the upper right quadrant) (**B**). No significant changes in the ratio of the mast cell population were found among the experimental groups. The experiment was performed with 3 mice per group. #, $p < 0.01$, ANOVA.



Supp. Figure 4. No inhibition by inotodiol of in vivo activation and proliferation of CD4⁺ DO11.10 TCR Tg T cells.

LN cells from DO11.10 TCR Tg mice were labeled with CFSE and adoptively transferred to BALB/C mice. The host mice were then immunized and treated with the respective reagents as described in Figure 5. Six days after immunization, cells were isolated from the whole –body LNs of the host mice and labeled with anti-CD4 plus anti-DO11.10 TCR mAbs, and analyzed with flow cytometry for the extents of CFSE dilution of CD4⁺DO11.10⁺ T cells. Non-proliferating CFSE-high (G1) and proliferating CFSE-intermediate to –low (G2) cells were gated separately as shown in Figure 5B and the ratio of the percentages of cells in G1 and G2 gates were calculated and plotted. #, $p < 0.01$, ANOVA.