Biochemical and Biophysical Research Communications 443 (2014) 266–271

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Perillyl alcohol suppresses antigen-induced immune responses in the lung

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ARTICLE INFO

Article history:
Received 19 November 2013
Available online 2 December 2013

Keywords:
Perillyl alcohol (POH)
Isoprenoid
Airway inflammation
Asthma

ABSTRACT

Perillyl alcohol (POH) is an isoprenoid which inhibits farnesyl transferase and geranylgeranyl transferase, key enzymes that induce conformational and functional changes in small G proteins to conduct signal production for cell proliferation. Thus, it has been tried for the treatment of cancers. However, although it affects the proliferation of immunocytes, its influence on immune responses has been examined in only a few studies. Notably, its effect on antigen-induced immune responses has not been studied. In this study, we examined whether POH suppresses Ag-induced immune responses with a mouse model of allergic airway inflammation. POH treatment of sensitized mice suppressed proliferation and cytokine production in Ag-stimulated spleen cells or $CD4^+$ T cells. Further, sensitized mice received aerosolized OVA to induce allergic airway inflammation, and some mice received POH treatment. POH significantly suppressed indicators of allergic airway inflammation such as airway eosinophilia. Cytokine production in thoracic lymph nodes was also significantly suppressed. These results demonstrate that POH suppresses antigen-induced immune responses in the lung. Considering that it exists naturally, POH could be a novel preventive or therapeutic option for immunologic lung disorders such as asthma with minimal side effects.

1. Introduction

The mevalonate pathway is a metabolic cascade which produces cholesterol. Cholesterol is intensively involved in cardiovascular diseases. Although cholesterol synthesis is thought to be conducted mainly in the liver, the mevalonate pathway is ubiquitous in living cells, and nonsterol products of this pathway also play an essential role in cell-signaling. Farnesyl diphosphate and geranylgeranyl diphosphate are intermediate metabolites of the mevalonate pathway, and are transferred to the C-terminal cysteines of target proteins by farnesyl transferase or by geranylgeranyl transferase, through farnesylation and geranylgeranylation, respectively [1]. Farnesylation and geranylgeranylation modulate various functions of proteins including the small GTPases Ras, Rac, and Ras homologue (Rho), by promoting the attachment of these proteins to cell membranes. For example, farnesylated Ras moves from the cytoplasm to cell membrane, then the activation of signal transduction pathways necessary for cell proliferation begins.

In normal sterologenic cells, there is a post-transcriptional feedback mechanism by which these nonsterol products down-regulate the overgrowth of cells through suppression of HMG-CoA reductase activity [2]. In contrast, tumor cells become resistant to this feedback mechanism, so up-regulation of HMG-CoA reductase results in continuous activation of the mevalonate pathway, which eventually leads to overgrowth of the cells [2]. Therefore, normalization of this pathway could become a novel strategy for cancer therapy. So far, several candidates including HMG-CoA reductase inhibitors (statins), inhibitors of mevalonic acid pyrophosphate decarboxylase, and inhibitors of farnesyl transferase, have been tried for treatment of cancers [2].

Among them, perillyl alcohol (POH) has attracted great attention. POH is a naturally occurring monoterpene, which exists in plants in a pure or mixed form. The mechanism by which POH suppresses cell growth has been studied intensively. POH acts as an inhibitor of farnesyl transferase [3,4] and geranylgeranyl transferase [5,6], which leads to suppress activities of various small GTPases [7,8]. Further, as these studies have confirmed that POH is relatively safe in terms of toxicity, POH has been considered a strong candidate for cancer treatment. In fact, phase I studies for

Abbreviation: POH, perillyl alcohol.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.11.106

refractory solid tumors have been reported [9,10]. Further, phase II trials have been conducted in prostate cancer [11], ovarian cancer [12], and metastatic colon cancer [13]. A recent study also showed that intranasal administration of POH (440 mg daily) increased the overall survival of patients with recurrent glioblastoma [14].

As POH suppresses the proliferation of cancer cells, it might affect the proliferation of immunocompetent cells as well. In addition, it might affect other functions of immnocompetent cells such as cytokine production and antibody production, thus modulating immune responses. So far, several in vitro studies have been conducted to examine the effect on T cells [15,16]. On the other hand, the effect of POH on immune cells in vivo has been examined only in a few studies with animal models of lupus [17], transplant coronary artery disease [17,18], and liver injury [19]. However, the effect on other types of immune responses, especially on allergic immune responses, has not been studied.

The purpose of the present study is to examine whether POH suppresses antigen-induced immune responses. We prepared a mouse model with OVA-induced allergic airway inflammation, and then examined the effect of POH.

2. Materials and methods

2.1. Mice

Male BALB/c mice 7 weeks of age were obtained from Charles River Japan (Kanagawa Japan). They were maintained under conventional animal housing conditions in a specific pathogen-free setting. All animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo (Tokyo, Japan).

2.2. Medium for cell culture

Complete DMEM was used as the medium for cell incubation. Complete DMEM consisted of DMEM with glucose (4.5 g/L), pyridoxine HCl (4.0 mg/l), and sodium pyruvate (110 mg/l), which contained FBS (10%, w/v), HEPES buffer sodium (0.01 M), MEM nonessential amino acid solution (0.1 mM), 2-ME (50 μM: Sigma–Aldrich), penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.3. Quantification of cytokines and cell proliferation

Cytokine concentrations in BALF, serum, and cell culture supernatants were determined by Enzyme-linked immunosorbent assay (ELISA). Concentrations of mouse IL-4, IL-5, IL-10, IFN-γ, IgE (BD pharmingen), and IL-13 (R&D Systems) were measured using commercial ELISA kits. We measured concentrations of mouse IL-17 by ELISA as previously reported [20]. Cell proliferation was measured by BrdU incorporation using a BrdU cell proliferation ELISA kit (Roche, Mannheim, Germany). The data were analyzed with Microplate Manager III, version1.45 (Bio-Rad).

2.4. Effects of POH on spleen cells in vitro

BALB/c mice were immunized with 2 μg of OVA (Sigma–Aldrich)/2 mg of alum i.p. on days 0. On day 10, spleen cells were obtained and cultured (2.5×10^6 cells/ml) with OVA (100 μg/ml) in the absence or presence of different concentrations of POH (0.1 or 1 μM) (Wako, Osaka, Japan). Then, cell proliferation and cytokine production were measured.

2.5. Preparation of single cell suspensions of spleen and lymph node cells

Spleens were collected and incubated at 37 °C for 15 min after treatment with 0.1% (w/v) collagenase (Sigma–Aldrich)/complete DMEM solution, then minced. Lymph node cells were collected and minced, then incubated at 37 °C for 30 min with 0.033% collagenase/complete DMEM solution. Single-cell suspensions were prepared by passing through a cell strainer. RBCs were removed by hypotonic lysis using RBC lysing buffer (Sigma–Aldrich). After two washes with HBSS, spleen or lymph node cells were used for experiments.

2.6. Spleen and lymph node cell responses to OVA

Spleen or lymph node cells (2.5×10^6/ml) were cultured in a 96-well, flat-bottomed microtiter assay plate with OVA (100 μg/ml) in an incubator (37 °C, 5% CO_2, 90% humidity). Cell Proliferation was measured on day 3. On day 4, cytokine production was measured by ELISA.

2.7. Animal preparation for ex vivo analyses

BALB/c mice were immunized with 2 μg of OVA/2 mg of alum i.p. on days 0 and 11. On days 11–17, mice were treated with POH (75 mg/kg body weight) dissolved in 0.5 ml of vehicle or the vehicle alone by i.p. injection. The vehicle for POH was Tricaprylin [17] (Sigma–Aldrich). The control mice received only saline injections on days 0 and 11. On day 18, spleens were collected and cell proliferation and cytokine production were measured as described above.

2.8. Effects of POH on $CD4^+$ T cells ex vivo

BALB/c mice were immunized with OVA/alum i.p. on days 0 and 11. On days 11–17, mice were treated with POH (75 mg/kg body weight) dissolved in 0.5 ml of vehicle or vehicle alone by i.p. injection. $CD4^+$ T cells were selected from spleen cells of the OVA-sensitized mice on day 18. To obtain $CD4^+$ T cells, we used monoclonal anti-mouse CD4 magnetic particles (BD Biosciences) following the manufacturer's protocol. The purity of $CD4^+$ cells, confirmed by flow cytometry, was >95%. Then $CD4^+$ cells (2.5×10^5/ml) were cocultured with freshly isolated mitomycin-C (Sigma–Aldrich)-treated splenocytes (2.5×10^5 cells/ml) under OVA stimulation (30 μg/ml). After 48 h, proliferation was assessed with a cell proliferation ELISA BrdU kit. After 72 h, the cytokine concentration in the supernatant was measured by ELISA.

2.9. Induction of allergic airway inflammation and POH treatment

BALB/c mice were immunized with OVA/alum i.p. on days 0 and 11. Control mice received i.p. injections of saline instead of the OVA/alum solution. Mice were challenged with an aerosolized solution of 3% w/v OVA in PBS for 10 min from day 18 to day 20. POH (75 mg/kg) dissolved in 0.5 ml of vehicle or the vehicle alone was given by i.p. injection from days 0 to 17. The control mice received PBS by inhalation on days 18–20. On day 21, samples of serum and bronchoalveolar lavage fluid (BALF) were obtained. The lungs were cut out and fixed with 10% neutralized buffered formalin. Thoracic lymph nodes were also obtained and used for analyses as described above. Cell counts and cell differentials in BALF were determined as previously reported [21]. Concentrations of IL-13 in BALF and IgE in sera were measured by ELISA.

2.10. Statistical analysis

Values are expressed as the mean ± SEM. Data were evaluated using a one-way ANOVA followed by the Student *t* test for comparisons between two groups. Values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Treatment with POH in vitro suppresses OVA-induced cytokine production in spleen

We first examined the effect of in vitro treatment with POH on Ag-induced immune responses. Treatment of spleen cells obtained from OVA-sensitized mice with POH attenuated the OVA-stimulated cytokine production in a dose-dependent manner (Fig. 1). On the other hand, POH did not affect cell proliferation in vitro (data not shown).

3.2. POH suppresses cell proliferation and cytokine production ex vivo

Next, we examined the effect of POH on cell activation ex vivo. Mice were sensitized with OVA/alum on days 0 and 11, and POH (75 mg/kg body weight) or vehicle was intraperitoneally injected on days 11–17. Control mice received only saline injections on days 0 and 11. On day18, spleen cells were collected from each group of mice, and cultured with OVA. Cell proliferation and cytokine production were significantly increased in splenocytes obtained from OVA-treated mice compared with those from saline-treated mice (Supplementary Fig. 1A–E). POH treatment significantly suppressed cell proliferation, although the effect was modest (Supplementary Fig. 1A). POH significantly downregulated the production of IL-5, IL-10, and IL-17, but IFN-γ was not affected (Supplementary Fig. 1B–E). So, POH suppressed Th2 and Th17 type cytokine production ex vivo.

3.3. POH suppresses proliferation and cytokine production in $CD4^+$ T cells

Next, we examined the effect of POH on $CD4^+$ T cells in the spleen. Mice were sensitized on days 0 and 11, and POH (75 mg/kg body weight) was administered by i.p. injection on days 11–17. On day 18, splenic $CD4^+$ T cells were stimulated with splenic APCs and OVA. POH attenuated the proliferation of $CD4^+$ T cells (Fig. 2A). In addition, it strongly suppressed IL-4, IFN-γ, and IL-17 production (Fig. 2B–D). These results showed that POH suppressed $CD4^+$ T cell priming during Ag sensitization ex vivo.

3.4. POH suppresses allergic airway inflammation

Based on the results of in vitro and ex vivo experiments, we examined whether POH affects OVA-induced airway responses in vivo. As expected, POH significantly decreased the number of eosinophils in BALF (Fig. 3A). In the histological analysis, POH also demonstrated a tendency to attenuate OVA-induced inflammation in the airway (Fig. 3B). The amount of IL-13 in BALF was also decreased by POH (Fig. 3C). On the other hand, POH did not downregulate total IgE production in sera (Fig. 3D), which suggested that POH exhibited its effects not through suppression of IgE production. Further, when POH was delivered during the OVA challenge, it did not significantly suppress airway inflammation (data not shown).

3.5. POH suppresses OVA-induced responses in MLN

At the time of sacrifice, thoracic lymph nodes were obtained, and the cell suspension was stimulated by OVA again. Treatment with POH in vivo during sensitization clearly attenuated cell proliferation of immunocytes in the thoracic lymph nodes (Fig. 4A). Further, it significantly suppressed IL-5 (Fig. 4B), IL-13 (Fig. 4C), IFN-γ (Fig. 4D), IL-17 (Fig. 4E) and IL-10 (Fig. 4F) production in lymph

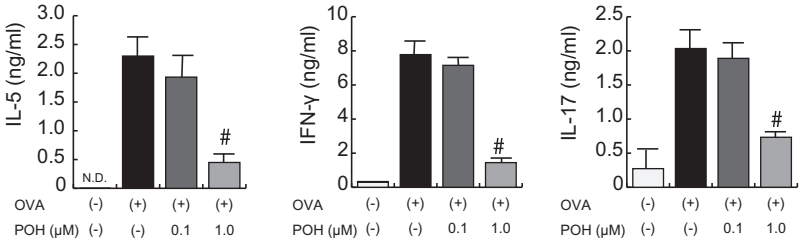

Fig. 1. POH suppresses Ag-induced cytokine production in vitro. Mice were sensitized with OVA/alum on day 0. On day 10, splenocytes were collected, and incubated with OVA (100 μg/ml) in the presence (0.1 or 1 μM) or absence of POH. After 72 h, the cytokine concentration in the supernatant was measured by ELISA. N.D., not detected, $^{\#}p < 0.05$ compared with cells treated with OVA in the absence of POH.

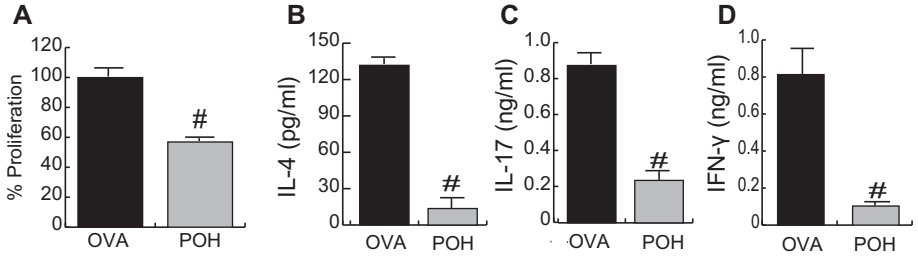

Fig. 2. POH treatment in vivo attenuates Ag-induced immune responses of $CD4^+$ T cells ex vivo. Mice were sensitized with OVA/alum on days 0 and 11. On days 11–17, they received intraperitoneal injections of POH (75 mg/kg/day) (POH), or vehicle (OVA). On day 18, $CD4^+$ T cells (2.5×10^5/ml) were positively selected by magnetic cell sorting, and cultured with freshly isolated mitomycin C-treated splenocytes (2.5×10^5 cells/ml) under OVA stimulation (30 μg/ml). (A) After 48 h, cell proliferation was assessed based on BrdU incorporation. Data are expressed as a percentage of the response compared with that of spleen cells from OVA mice. (B–D) Production of interleukin (IL)4 (B), IL-17 (C), and IFN-γ (D) was measured after 72 h of incubation with OVA. $^{\#}p < 0.05$ compared with the value for OVA-treated mice.

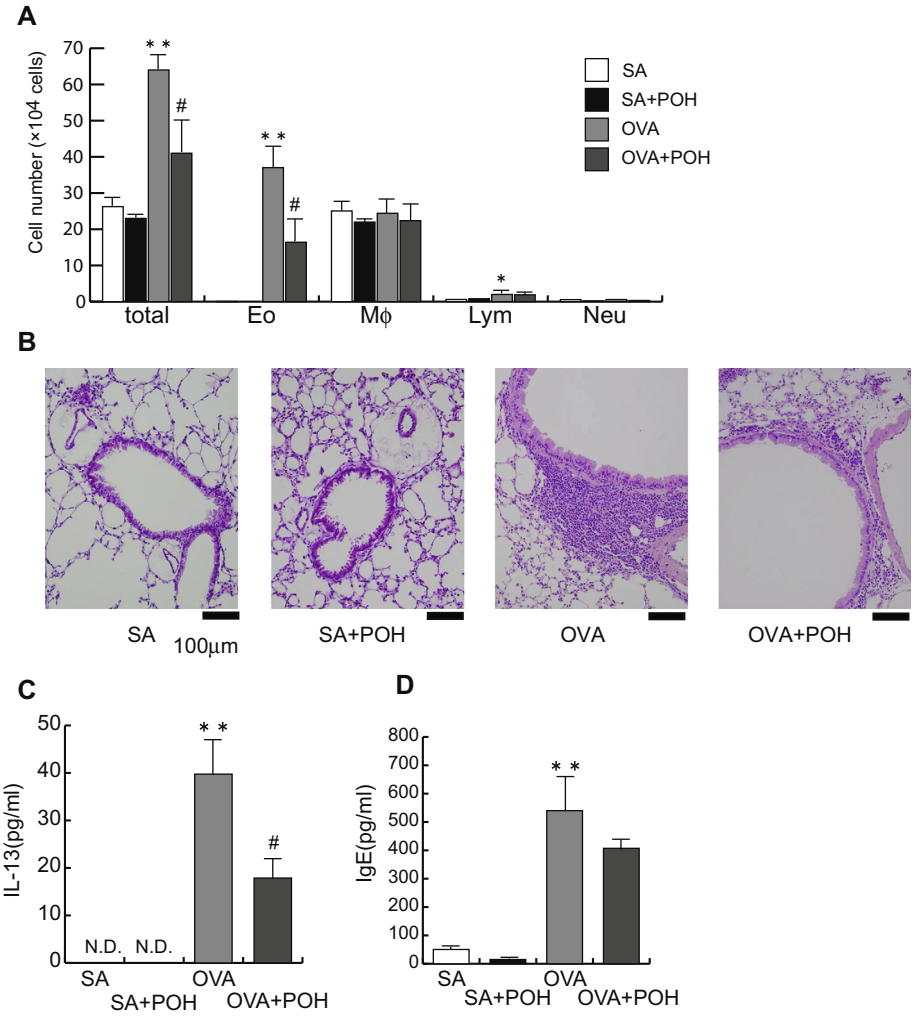

Fig. 3. POH suppresses Ag-induced airway inflammation. Mice were sensitized with OVA on days 0 and 11. Then, they were challenged with an OVA aerosol on days 18–20. On days 0–17, the mice received intraperitoneal injections of POH (75 mg/kg/day) or vehicle. Control mice received saline injections on days 0 and 11, and a phosphate-buffered saline (PBS) aerosol on days 18–20. On day 21, bronchoalveolar lavage fluid (BALF), lung tissues, and serum were obtained. Mice with sensitization (i.p.)/administrations (i.p.)/challenges (nebulization) are represented as follows: saline/vehicle/PBS, SA; saline/POH/PBS, SA + POH; OVA/vehicle/OVA, OVA; OVA/POH/OVA, OVA + POH. (A) Cell differentials in BALF. (B) Hematoxylin eosin staining of lung tissue (scale bar 100 μm). (C) Interleukin (IL)13 concentrations in BALF. (D) Total IgE levels in serum. N.D; not detected. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the value for SA mice. $^{\#}p < 0.05$ compared with the value for OVA mice.

nodes. These results clearly indicated that POH suppressed OVA-induced immune responses also in the thoracic lymph nodes.

4. Discussion

The results of the present study clearly demonstrated that POH attenuates Ag-induced immune responses in the lung. It suppressed OVA-induced cytokine production by spleen cells in vitro. POH, delivered during sensitization, also suppressed OVA-induced T cell priming in spleen ex vivo. Further, POH attenuated OVA-induced airway eosinophilia and production of Th2 cytokines in the lung and thoracic lymph nodes. As far as we know, this is the first study to confirm an immunosuppressive effect of POH on allergic inflammation.

Several studies in vitro have elucidated the effect of POH on immune responses. Schulz et al. reported that POH and its derivative d-limonen suppressed lymphocyte proliferation [15]. They also reported that perillic acid, a metabolite of POH, reduced IL-2 production in T lymphocytes by inhibiting Ras/MAP kinase activity [22]. In another report, POH inhibited T cell receptor-mediated calcium ion signaling [16]. These findings raise the possibility that POH or its derivative might suppress many kinds of immune responses in vivo. Nevertheless, very few studies have examined this possibility. In an animal model of lupus, Liu et al. found that the intraperitoneal administration of POH inhibited production of autoantibodies, a result also confirmed in vitro [17]. On the other hand, the effect of POH on Ag-induced immune responses has not been studied, which drove us to conduct the current study.

In the present study, we applied the same dosage of POH (75 mg/kg body) as used in previous studies on lupus mice [17]. This dosage is lower than that which induces severe side effects or toxicity in humans [2,23]. In fact, we did not observe any apparent side effects in our experimental mice. We sequentially analyzed spleen cells by staining with annexin V and PI, and confirmed that the suppressive effect of POH was not due to cytotoxicity among immunocompetent cells (data not shown). As compared to other anti-cancer drugs that inhibit the mevalonate pathway, a characteristic feature of POH is its minimal side effects [2]. Phase I [9,10] and II [11–13] studies of POH have revealed dose-limiting toxic effects including nausea, vomiting, anorexia, unpleasant taste, and eructation. The maximum tolerated dose was determined to be 8.4 g/M^2/day delivered orally in four parts [23]. So, POH could be applied to humans with minimal side effects.

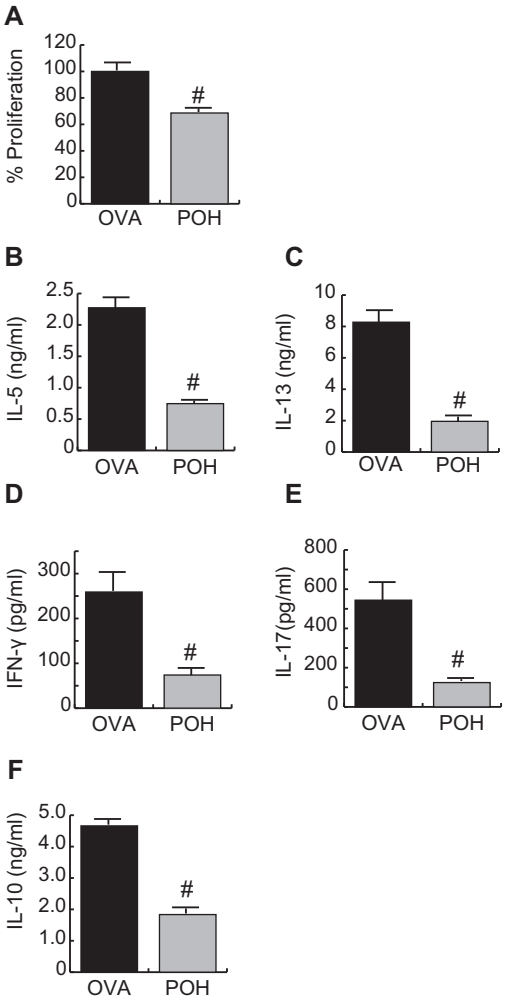

Fig. 4. Treatment with POH suppresses cytokine production in LN. On day 21, lymph node cells from OVA-injected mice treated with POH (POH) or vehicle (OVA) were incubated with OVA. After 3 days of incubation, cell proliferation (A) and production of IL-5 (B), IL-13 (C), IFN-γ (D), IL-17 (E) and IL-10 (F) were measured. Data were obtained from six wells per group of mice. $^{\#}p < 0.05$ compared with the value for OVA-treated mice.

In the present study, we clarified that POH inhibits proliferation and cytokine production in Ag-stimulated $CD4^+$ T cells (Fig. 2). However, its mechanism of action in T cells was not fully studied. In addition, whether POH actually down-regulates the prenylation of small G proteins such as Rho and Ras in vivo remains to be elucidated. Further understanding of the precise mechanisms of action of POH in vivo would help us consider the clinical application of this compound to various immune-mediated disorders. In addition, effects on other types of immunocompetent cells such as dendritic cells, mast cells, and eosinophils, or on fibroblasts, should be studied further.

Statins are another kind of drug that inhibits the mevalonate pathway. It is well established that statins possess immunomodulatory effects [1]. Statins are now considered as a kind of immunomodulatory drug, and are a candidate drug for the treatment of immunological disorders such as multiple sclerosis and auto immune diseases [1]. There are several reports including ours that demonstrate a suppressive effect of statins on experimental allergic airway inflammation [20,24]. Further, an anti-cancer effect of statins has also been proposed, although this is still controversial [2]. Generally, the suppressive effect of statins on cell proliferation is not as strong as that of POH, an anti-cancer drug. Therefore, the suppressive effect of POH on immunocompetent cells might be stronger than that of statins, so the effect on immune responses would be also stronger. At present, the effect of statins on patients with allergic bronchial asthma has not been clinically confirmed. POH or its derivatives could be effective for the treatment of asthma.

POH exists naturally in plants in a pure or mixed form. Further, its derivative, limonene, is included in natural foods such as lemons and oranges and has a similar biological effect to POH [15,25]. Consequently, POH and its derivatives could be a novel candidate for the treatment of asthma, although a better understanding of their mechanisms of action would be needed before any clinical application. For example, they could be used to control moderate to mild asthma and help reduce the dosage or allow the discontinuation of conventional anti-asthma drugs. A second possible application might be in the prevention of allergic disorders by eating lemons or oranges for a certain period of time. On the other hand, the administration of POH during airway OVA challenges did not suppress airway inflammation, suggesting that POH would not be effective against established asthma. To clarify these issues, clinical trials and epidemiological studies will be needed.

In summary, we demonstrated that POH suppressed Ag-induced systemic immune responses and attenuated allergic airway inflammation in a mouse model. Considering that it exists in nature, POH or its derivatives could be a novel preventive or therapeutic option for asthma with minimal side effects. To achieve this goal, more studies on its mechanisms of action should be conducted.

Conflict of interest

There is no conflict of interest.

Acknowledgments

We thank K. Kurosaki for technical assistance.

This work was supported by JSPS KAKENHI Grant Number 23591137.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.106.

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