



Diet containing a polyphenol concentrate from pomegranate juice attenuates contact hypersensitivity in mice

Takao Nagano^{a,b,*}, Hideyuki Ito^c

^a Department of Food Science, Faculty of Bioresources and Environmental Sciences, Ishikawa Prefectural University, 1-308 Suematsu, Nonoich, Ishikawa 921-8836, Japan

^b Department of Clinical Nutrition, Faculty of Health Science and Technology, Kawasaki University of Medical Welfare, 288, Matsushima, Kurashiki, Okayama 701-0193, Japan

^c Department of Nutritional Science, Faculty of Health and Welfare, Okayama Prefectural University, 111 Kuboki, Soja, Okayama 719-1197, Japan

ABSTRACT

Murine contact hypersensitivity (CHS) is an animal model of allergic contact dermatitis (ACD). We investigated the inhibitory effects of dietary pomegranate polyphenol concentrate (PPC) on CHS. Mice were fed diets containing low or high doses of PPC for 3 weeks prior to CHS induction. Both the low- and high-dose PPC diets attenuated ear swelling and edema, and resulted in reduced infiltration of Gr-1-positive myeloid cells into ear tissues. The ear tissues of PPC-treated mice exhibited reduced mRNA expression of *Cxcl2* and *Cxcl3* and decreased production of chemokine (C-X-C motif) ligand 2 (CXCL2) and chemokine (C-C motif) ligand 12 (CCL12). Furthermore, the effects of urolithin A (UA) on splenic cell activation were studied. UA reduced the number of INF- γ -producing cells. These results indicate that low doses of dietary PPC alleviate CHS symptoms, suggesting that dietary PPC may provide beneficial effects on ACD at physiologically relevant doses to humans.

1. Introduction

Pomegranates (*Punica granatum* L.) are widely consumed as fresh fruit and as beverages. Notably, pomegranate juice (PJ) contains a higher concentration of total polyphenols (0.2%–1.0%), consisting primarily of anthocyanins, ellagitannins (ETs), and gallic and ellagic acids, than other fruit juices (Aviram & Rosenblat, 2012; Bakkalbaşı, Menteş, & Artik, 2008). Commercial PJ also exhibits three-fold higher antioxidant activity than red wine and a green tea. While ETs comprise the predominant antioxidant compounds in PJ, anthocyanins and ellagic acid derivatives also contribute to the total antioxidant capacity of the juice (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000). Such high antioxidant activities have been associated with beneficial health effects, thereby suggesting that PJ might exert preventive effects against chronic diseases such as cancer, diabetes, cardiovascular diseases, and inflammatory bowel diseases (Faria & Calhau, 2011; Heber, 2008; Larrosa, Garcia-Conesa, Espin, & Tomas-Barberan, 2010). The bioavailability of ETs from PJ has been studied in human subjects. The ETs were hydrolyzed to yield ellagic acid (EA), which was further metabolized in the gastrointestinal tract by the colon microflora to urolithin derivatives (mainly urolithin A, UA). These polyphenols were well absorbed, whereas the absorption of intact ETs and EA is very low (Espin, Larrosa, Garcia-Conesa, & Tomas-Barberan, 2013).

Allergic contact dermatitis (ACD) is one of the most common skin diseases caused by delayed-type hypersensitivity to antigens that come into contact with the skin, and the mouse contact hypersensitivity (CHS) model comprises a commonly used experimental animal model for studying human ACD (Honda, Egawa, Grabbe, & Kabashima, 2013; Kaplan, Igyártó, & Gaspari, 2012; Martin et al., 2011). In previous work, we prepared a pomegranate polyphenol concentrate (PPC) from pomegranate juice and investigated its inhibitory effects on dinitrofluorobenzene (DNFB)-induced CHS in mice. PPC diets significantly inhibited DNFB-induced ear swelling and reduced antigen-specific immunoglobulin (Ig)G1 production. PPC diets also resulted in increased numbers of splenic interleukin (IL)-10- and interferon (IFN)- γ -producing CD4⁺ T helper (Th) cells. These results suggest that PPC diets exert a protective effect against CHS and IgG1 production by increasing the number of IL-10-producing CD4⁺ T cells and modulating Th1/Th2 balance (Nagano, Nishida, & Ito, 2018). Notably, however, the amount of polyphenols consumed by these PPC mice was not physiologically relevant to humans.

In this study, to address this issue, we examined the effects of diets containing low doses of PPC on CHS. Furthermore, to clarify the mechanism underlying the immune-regulatory effects of PPC, we evaluated the numbers of infiltrating myeloid immune cells and the production of chemokines in contact allergic ear tissues.

* Corresponding author at: Department of Food Science, Faculty of Bioresources and Environmental Sciences, Ishikawa Prefectural University, 1-308 Suematsu, Nonoich, Ishikawa 921-8836, Japan.

E-mail address: naganot@ishikawa-pu.ac.jp (T. Nagano).

<https://doi.org/10.1016/j.jff.2018.04.038>

Received 18 December 2017; Received in revised form 24 March 2018; Accepted 16 April 2018

Available online 21 April 2018

1756-4646/ © 2018 Elsevier Ltd. All rights reserved.

2. Materials and methods

2.1. Material

PPC was prepared as previously described (Nagano et al., 2018). The phenolic content of the concentrate was 59.5%, as determined via the Folin-Denis method using ethyl gallate as a standard. The detailed polyphenolic compounds in PPC were reported previously (Ito et al., 2014; Kawakami, Li, Uraji, Hatanaka, & Ito, 2014).

2.2. Animals

Six-week-old female BALB/c mice (15–20 g body weight) were purchased from Japan SLC (Hamamatsu, Japan). The animals were fed a soybean-free diet (F2PLD1; Oriental Yeast, Tokyo, Japan) and housed in a temperature-controlled room ($25 \pm 2^\circ\text{C}$) with a 12-h light/dark cycle. All experiments were designed in accordance with the guidelines for animal experimentation, and were approved by the Animal Experimental Committee of Kawasaki University of Medical Welfare (authorization number 13-011) and Kawasaki Medical School (authorization number 14-019).

2.3. Contact hypersensitivity test

Mice were divided into four groups according to the diet they received: the non-CHS control group ($n = 3$), the CHS control group ($n = 6$), the low-dose PPC group ($n = 6$), and the high-dose PPC group ($n = 6$). The non-CHS and CHS control groups were provided acidified water, while the low- and high-dose PPC groups were provided water supplemented with 0.01% and 0.2% PPC, respectively, via drinking water for three weeks prior the first DNFB challenge. The mice in the CHS and PPC groups were sensitized on days 0 and 1 by applying 50 μL of 0.5% DNFB (Kanto Chemical, Tokyo, Japan) diluted in acetone/olive oil (4:1) to shaved abdominal skin, and then challenged on days 5 and 12 by application of 20 μL of 0.2% DNFB to the dorsal side of both ears. Negative control (non-CHS) mice were sensitized and challenged with vehicle lacking DNFB. Ear thickness was measured using a soft touch micrometer (CLM1-15QM; Mitutoyo, Tokyo, Japan) and ear swelling was calculated as the difference in ear thickness before the initial DNFB challenge and at 24 h after the final challenge. All experimental procedures were performed under anesthesia with sevoflurane (Maruishi Pharmaceutical, Osaka, Japan).

2.4. Histological analysis

Ear tissue was harvested at 24 h after the second DNFB challenge, fixed with IHC Zinc fixative (BD Biosciences, San Jose, CA, USA) for 24 h, embedded in paraffin, and sectioned. Tissue sections were then stained with hematoxylin and eosin and analyzed by immunohistochemistry (IHC) using a Ventana Discovery® XT instrument (Ventana Medical Systems, Tucson, AZ, USA), as described previously (Nagano et al., 2016); anti-mouse Ly-6G (Gr-1) purified (1:25; eBioscience, San Diego, CA, USA) and biotin mouse anti-rat IgG2b (1:25; BD Biosciences) were utilized as the primary and secondary antibodies, respectively. Images were acquired using an Axio Photo microscope (Carl Zeiss AG, Oberkochen, Germany) and the number of Gr-1-positive cells was enumerated in five randomly selected areas ($\sim 210 \mu\text{m}^2$) of each tissue section.

2.5. Gene expression assay

Ear tissues of mice were collected at 24 h after the second challenge with DNFB. Total RNA was extracted from the ear tissues using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Venlo, Netherlands) and subjected to DNA microarray analysis, which was performed by Filgen, Inc. (Nagoya, Japan), using a GeneChip Mouse Gene 2.0 ST Array

(Affymetrix, Santa Clara, CA, USA). Total RNA was reverse-transcribed using the ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan), and the mRNA expression levels of *Cxcl3*, *Cxcl2*, and *Ccl3* were determined for each sample by real-time PCR analysis using TaqMan gene expression assays (Applied Biosystems, Waltham, MA, USA) and THUNDERBIRD probe qPCR mix (Toyobo) with a StepOnePlus™ system (Applied Biosystems), according to the manufacturers' protocols. β -Actin was used as a control for normalization, and all assays were performed in triplicate. TaqMan gene expression assays (Applied Biosystems) were used for the following genes: *Cxcl2* (Mm00436450_m1), *Cxcl3* (Mm01701838_m1), *Ccl3* (Mm00441259_g1), and *Actb* (Mm00607939_s1). Fluorescence data were analyzed using the StepOnePlus™ software and were expressed as C_t , the number of cycles needed to generate a fluorescent signal above a predefined threshold.

2.6. Chemokine production assay

Ear tissues were collected at 24 h after the second DNFB challenge and were stored at -80°C before use. The ears were cut, placed into Lysing Matrix D tubes, and homogenized in PIPA lysis buffer containing protease inhibitors (ATTO, Tokyo, Japan) in a FastPrep-24 device (MP Biomedicals, Santa Ana, CA, USA). Protein concentrations were determined in duplicate using the Pierce™ BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA), and chemokine expression levels were analyzed using the Proteome Profiler™ mouse chemokine array kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Chemiluminescence was detected using an ImageQuant LAS 4000 mini instrument (GE Healthcare Biosciences, Piscataway, NJ, USA), and spot intensity was quantified with Image Studio Lite software (LI-COR Biotechnology, Lincoln, NE, USA). The expression levels of chemokine (C-X-C motif) ligand 2 (CXCL2) and chemokine (C-C motif) ligand 12 (CCL12) were determined using a mouse CXCL2/MIP-2 and CCL12/MCP-5 DuoSet enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems), according to the manufacturer's instructions. All assays were performed in duplicate.

2.7. Intracellular cytokine staining

The CHS control mice were scarified under anesthesia on day 13. The spleen was immediately excised and splenic single cells were suspended in RPMI-1640 medium (Sigma-Aldrich) containing 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich). The cells (1×10^7 cells/well) were stimulated in 6-well plates for 5 h with 50 ng/mL 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin in the presence of protein transport inhibitor (containing monensin, BD Bioscience) at 37°C under a humidified atmosphere containing 5% CO_2 . UA was added 10 min before the cell stimulation in experiments in which its effects were investigated. After the stimulation, the cells were counted and their viability was assessed with a trypan blue exclusion assay. The stimulated cells were fixed with BD fixation buffer (BD Bioscience), permeabilized with BD perm/wash buffer (BD Bioscience), and stained with fluorescence-labeled antibodies against the respective intracellular cytokine (IFN- γ , IL-4, or IL-10) expressed on the cell surface according to the manufacturer's instruction. The stained cells were analyzed using a BD FACSCalibur™ (BD Bioscience).

2.8. Statistical analysis

Data are presented as means $\pm$ standard errors of the mean (SEM). Statistical significance was calculated via one-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests, using Origin 8.5 software (OriginLab, Northampton, MA, USA). $P < 0.05$ was considered statistically significant for all tests.

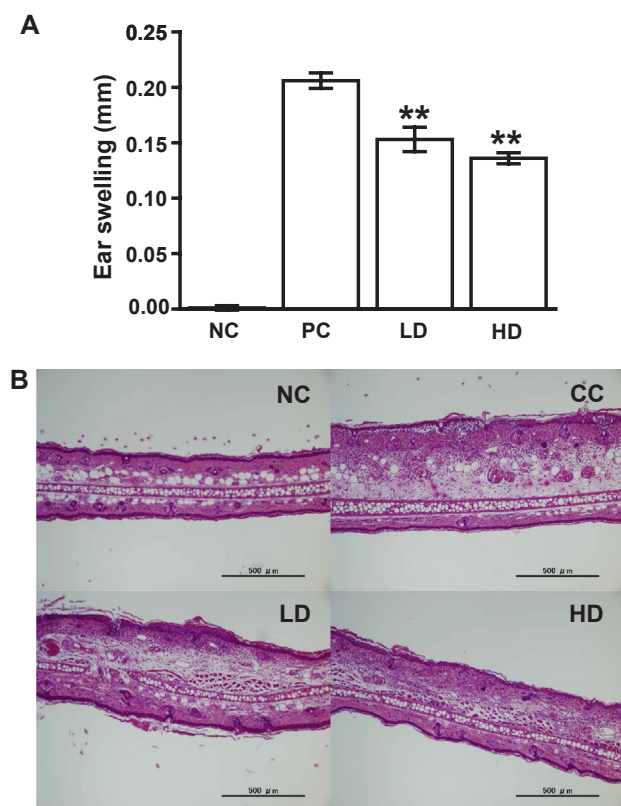


Fig. 1. Dietary pomegranate polyphenol concentrate (PPC) reduces ear swelling and edema in mice with contact hypersensitivity (CHS). (A) Ear swelling. Ear thickness was measured before and at 24 h after the second challenge with dinitrofluorobenzene (DNFB), and ear swelling was calculated as the difference in ear thickness between these two time points. NC, non-CHS control group ($n = 3$); CC, CHS control group ($n = 6$); LD, low-dose PPC-treated group ($n = 6$); HD, high-dose PPC-treated group ($n = 6$). The data are presented as the means $\pm$ standard errors of the mean (SEM) of the data from three independent experiments; $^{**}P < 0.01$ vs. CC. (B) HE-stained images of ear tissues. Ear tissues were collected at 24 h after the second challenge with DNFB, fixed with zinc for 24 h, embedded in paraffin, and sectioned. Ear tissue sections were then subjected to hematoxylin and eosin (HE) staining. Scale bars, 500 μ m.

3. Results

3.1. Dietary PPC attenuates DNFB-induced CHS

The suppressive effect of PPC-enriched diets on CHS was evaluated by measuring ear swelling at 24 h after the second DNFB challenge (Fig. 1A). Compared to the untreated CHS group (0.206 ± 0.007 mm), the low-dose PPC-treated (0.153 ± 0.011 mm) and high-dose PPC-treated (0.136 ± 0.005 mm) mice exhibited significantly reduced ear swelling (by 25.7% and 34.0%, respectively; $P < 0.01$), indicating that dietary PPC attenuates CHS symptoms. Histopathological analysis of these inflamed ear tissues revealed prominent tissue edema and increased infiltration of immune cells in the CHS mice compared to the non-CHS mice, which was alleviated by both PPC-enriched diets (Fig. 1B). In addition, we assessed the level of infiltration of myeloid immune cells into the ear tissues of CHS mice by staining for Gr-1 antigen, a marker of granulocytes and monocytes typically recruited in large numbers to the site of allergen challenge during the effector phase (Fig. 2A). There was a significantly greater decrease in the number of infiltrated Gr-1-positive cells in the contact allergic ear tissues of the mice in the low-dose PPC (253 ± 21 cells/mm²; 27.2% decrease; $P < 0.01$) and high-dose PPC treatment (221 ± 26 cells/mm²; 36.1% decrease; $P < 0.01$) groups than in those of the untreated CHS group

(346 ± 25 cells/mm²) (Fig. 2B). These results suggest that both high-dose and low-dose dietary PPC yield anti-inflammatory effects in CHS mice.

3.2. Dietary PPC inhibits the mRNA expression of *Cxcl3* and *Cxcl2* in mice with CHS

To clarify the mechanism through which dietary PPC attenuates CHS, we examined the mRNA expression levels of cytokine genes in the inflamed ear tissues of high-dose PPC-treated and untreated CHS mice at 24 h after the second challenge with DNFB via DNA microarray analysis. The greatest decreases in mRNA expression among cytokine genes in the high-dose PPC-treated mice were observed for *Cxcl3*, *Cxcl2*, *Ccl3*, *Ifng*, and *Il1b* (Table 1). Real-time PCR analysis was then utilized to confirm the mRNA expression levels of *Cxcl3*, *Cxcl2*, and *Ccl3* (Fig. 3). While the ear tissues from both the low-dose and high-dose PPC-treated mice exhibited significantly lower expression of *Cxcl3* and *Cxcl2* than those of the untreated CHS mice ($P < 0.05$), there was no significant difference in *Ccl3* expression among the PPC-treated and untreated mice. These results demonstrate that PPC diets result in reduced expression of the *Cxcl3* and *Cxcl2* genes in contact allergic ear tissues.

3.3. Dietary PPC suppresses CXCL2 and CCL12 secretion in mice with CHS

To further improve our understanding of the suppressive effects of PPC diets on the production of chemokines at the elicitation sites of CHS, we utilized a chemokine antibody array to examine the expression levels of 25 chemokines in the inflamed ear tissues of mice in each group at 24 h after the second DNFB challenge. Compared to the untreated CHS mice, animals receiving the high-dose PPC diet exhibited decreased release of the chemokines CXCL5, CCL12, CCL5, CXCL12, and CXCL2 (Table 2). Moreover, ELISA analysis confirmed that the ears of mice in the low- and high-dose PPC groups exhibited reduced CXCL2 and CCL12 expression compared to those of the mice in the untreated CHS group ($P < 0.05$; Fig. 4). These findings indicate that dietary supplementation with PPC can markedly suppress the release of the pro-inflammatory mediators CXCL2 and CCL12 at allergic reaction sites in mice with CHS.

3.4. UA modulates the activated splenic cell

The cell viability at different concentrations of UA was measured to determine whether the compound was toxic to splenic cells (Fig. 5A). UA did not significantly affect cell viability of the splenic cells at concentrations less than 40 μ M. The effects of UA on activated splenic cells were investigated at different concentrations of UA (Fig. 5B). The number of IFN- γ -producing cells was decreased with increasing concentrations of UA. However, the number of IL-4- and IL-10-producing cells was not significantly affected by the different UA concentrations.

4. Discussion

The results of this study clearly demonstrate that low-dose PPC diets suppressed CHS in mice. Specifically, we observed attenuated edema corresponding to the inhibition of inflammation in the ear of CHS mice, as evidenced by the reduced infiltration of Gr-1-positive immune cells and the downregulation of pro-inflammatory mediators such as CXCL2 and CCL12, in PPC-treated mice compared to control mice. In our previous study, mice were fed only the high-dose of PPC, resulting in high levels of polyphenol-intake (Nagano et al., 2018). Our PPC preparation contained 59.5% phenolics, and the measured average daily intake of the PPC solution was 4–5 g per mouse. As such, each mouse receiving this high dose consumed 5–6 mg of polyphenols per day, or approximately 300 mg polyphenols/kg bodyweight/day, which corresponds to approximately 20 g per day for a 60 kg human. For

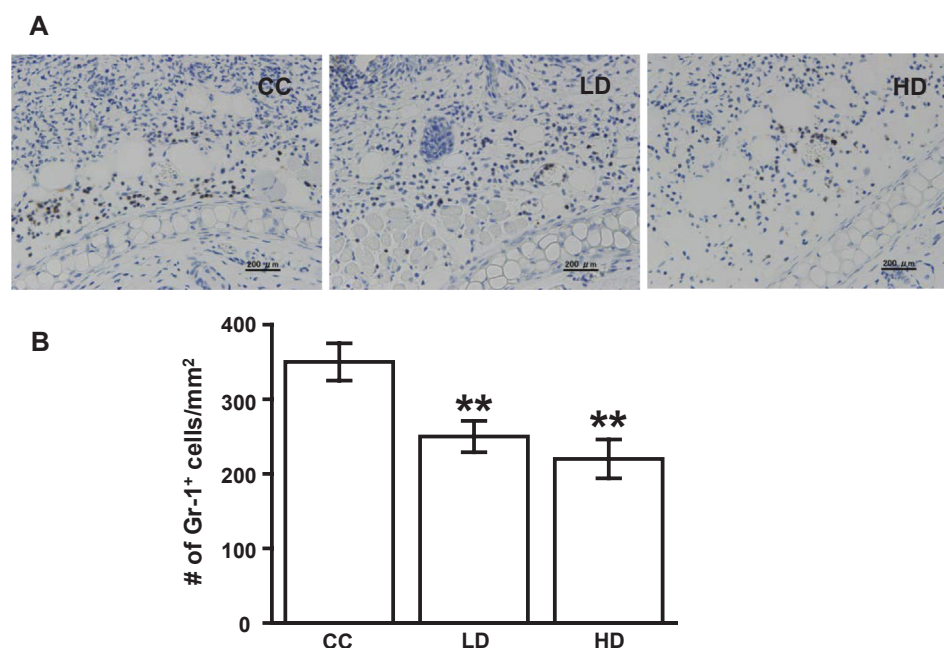


Fig. 2. Dietary pomegranate polyphenol concentrate (PPC) reduces the infiltration of myeloid cells in contact hypersensitivity (CHS) mice. Ear tissue sections were obtained as described in Fig. 1 legend and then subjected to immunohistochemical (IHC) staining using an anti-Gr-1 antibody. (A) IHC images of ear tissues. Dark-brown, Gr-1-positive cells; blue, nuclei stained with hematoxylin. CC, contact hypersensitivity (CHS) control group; LD, low-dose pomegranate polyphenol concentrate (PPC)-treated group; HD, high-dose PPC-treated group. Scale bars, 200 μm. (B) the number of Gr-1-positive cells infiltrating the ear tissues of mice in each group. The data are presented as the means ± standard errors of the mean (SEM) of the data obtained from three independent experiments (n = 6 mice per group); **P < 0.01 vs. CC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Cytokine genes that were down-regulated in mice receiving the high-dose PPC diet, compared with the untreated CHS control mice, as determined by DNA microarray analysis.

Gene symbol	Expression value		Ratio
	High-dose PPC	CHS control	
<i>Cxcl3</i>	44	121	0.37
<i>Cxcl2</i>	79	140	0.55
<i>Ccl3</i>	340	509	0.62
<i>Ifng</i>	47	67	0.72
<i>Il1b</i>	827	1171	0.74

PPC, pomegranate polyphenol concentration; CHS, contact hypersensitivity.

comparison, polyphenol content of PJ varies between 0.2 and 1% (Martin et al., 2011), which amounts to 0.4–2 g polyphenols per 200 mL juice. Thus, the low PPC dose administered to the mice corresponds to approximately 1 g polyphenols for a human per day. As such, the findings presented here indicate that a glass of PJ per day may be sufficient to yield beneficial effects in individuals with ACD.

In this study, DNA microarray analysis detected reduced expression of the *Ifng* and *Il1b* genes in high-dose PPC-treated mice compared to untreated CHS mice. IFN-γ and IL-1β are pro-inflammatory cytokines that play critical roles in eliciting CHS (Grabbe & Schwarz, 1998; Honda et al., 2013). Indeed, since this reaction is mediated by IFN-γ-producing Th1 and CD8⁺ T cytotoxic (Tc) 1 cells, high levels of IFN-γ and an accumulation of Th1/Tc1 cells have been observed at the elicitation sites (Grabbe & Schwarz, 1998). Meanwhile, IFN-γ knockout (KO) mice failed to elicit CHS upon treatment with urushiol. Urushiol-induced CHS and the corresponding mRNA expression of IFN-γ were also remarkably suppressed in mice treated with monoclonal antibodies against CD4 or CD8 (Wakabayashi et al., 2005). In another study, Mori et al. (2008) examined the CHS response to picryl chloride (PCI) in IFN-γ KO mice. The attenuation of PCI-induced CHS observed in that study corresponded with reduced inflammation in the ear tissues, as evidenced by reduced infiltration of CD3-positive immune cells (Mori et al., 2008). The elicitation of CHS responses can be explained using both antigen-nonspecific and -specific inflammatory signals (S. Grabbe et al., 1996). In antigen-nonspecific inflammation, haptens stimulate keratinocytes to produce pro-inflammatory cytokines such as IL-1β and

tumor necrosis factor (TNF)-α. In a previous study, Shornick et al. (1996) demonstrated that IL-1β-deficient mice exhibited defective CHS responses to the hapten trinitrochlorobenzene (TNCB) (Shornick et al., 1996). Moreover, CHS responses are dependent on the NLRP3 inflammasome and on IL-1 receptor (IL-1R) signaling. The NLRP3 inflammasome activates caspase-1 which cleaves the inactive preforms of IL-1β. Mice deficient in NLRP3 and the adaptor protein ASC showed impaired CHS responses to trinitrophenylchloride (Sutterwala et al., 2006). Similar effects were observed in caspase-1- and IL-1R-deficient mice and in mice treated with an IL-1R antagonist (Watanabe et al., 2007).

The infiltration of inflammatory cells into ear tissues during CHS arises from the sequential and coordinated expression of chemokine genes (Mitsui et al., 2003; Niwano, Mitsui, & Kohno, 2008). In a previous study, the expression levels of chemokine genes were examined at the elicitation site of TNCB-induced CHS BALB/c mice. The expression levels of the 11 genes examined were assigned to four groups based on their kinetic patterns. At the early time point, there was increased expression of neutrophil-recruiting chemokines such as CXCL1 and CXCL2. Meanwhile, the expression levels of chemokines in the monocyte chemoattractant protein (MCP) group, including CCL2 and CCL12, increased over time, reaching maximum levels at 6–9 h post-challenge (Mitsui et al., 2003). In this study, we observed decreased expression of *Cxcl2* and *Cxcl3* and reduced production of CXCL2 and CCL12 in PPC-treated mice compared to untreated CHS mice. Moreover, the ear tissues of PPC-fed mice exhibited reduced infiltration of Gr-1-positive myeloid cells. Therefore, the observed suppressive effects of PPC diets on the expression of CXCL2 and CCL12 correlates with reduced infiltration of neutrophils and monocytes into the contact allergic ear tissues.

Several studies have demonstrated that certain topical treatments inhibit the gene and protein expression of chemokines such as CXCL2 and CCL12 at the elicitation sites of CHS mice (Honda et al., 2009; Mitsui et al., 2004; Nakamura et al., 2016). Mitsui et al. (2004) demonstrated that topical difluortolone valerate treatment suppressed ear swelling and inflammatory cell infiltration in TNCB-induced CHS mice within 24 h after the TNCB challenge, and yielded reduced expression of each of the eight chemokine genes tested, including CXCL2 and CCL12, compared to the untreated mice (Mitsui et al., 2004). Meanwhile, Honda et al., 2009 showed that blockade of EP3, one of the four subtypes of prostaglandin E₂ receptor, with the selective antagonist

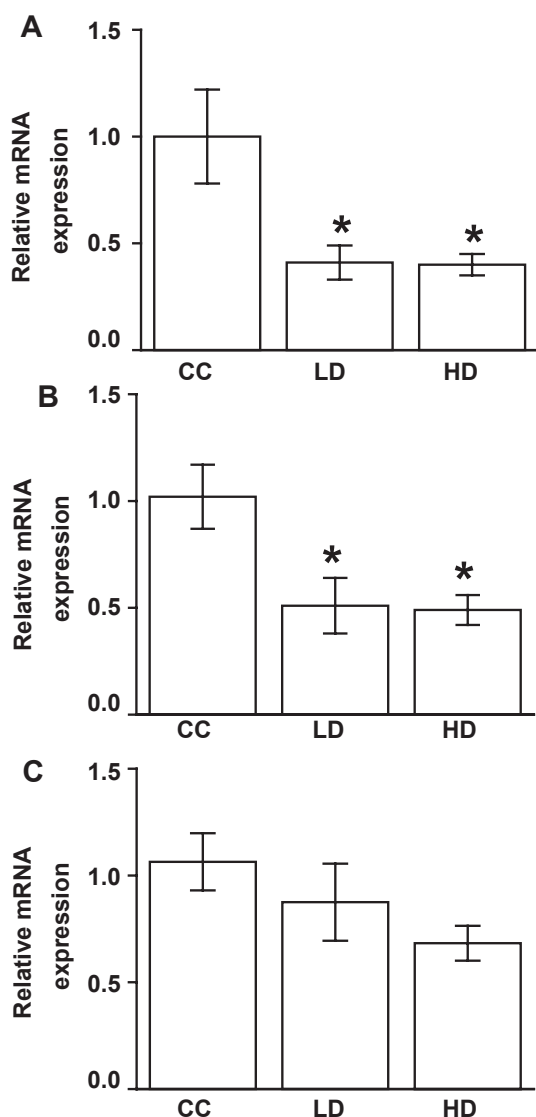


Fig. 3. Dietary pomegranate polyphenol concentrate (PPC) inhibits the expression of the *Cxcl2* and *Cxcl3* genes in mice with contact hypersensitivity (CHS). Ear tissues were collected at 24 h after the second challenge with dinitrofluorobenzene. The mRNA expression levels of (A) *Cxcl2*, (B) *Cxcl3*, and (C) *Ccl3* were then determined by real-time PCR analysis. *Actb* (β -Actin) was used as a control for normalization. CC, CHS control group; LD, low-dose PPC treated group; HD, high-dose PPC-treated group. The data represent the results of two independent experiments ($n = 6$ mice per group) and are presented as means $\pm$ standard errors of the mean (SEM); * $P < 0.05$ vs. CC.

Table 2

Chemokines that were down-regulated in mice receiving the high-dose PPC diet, compared with the untreated CHS control mice, as determined by antibody array.

Cytokine	Fluorescence intensity		Ratio
	High-dose PPC	CHS control	
CXCL5	9100	20,500	0.44
CCL12	36,200	69,200	0.52
CCL5	10,900	18,900	0.58
CXCL12	15,100	21,300	0.71
CXCL2	4300	5700	0.76

PPC, pomegranate polyphenol concentration; CHS, contact hypersensitivity.

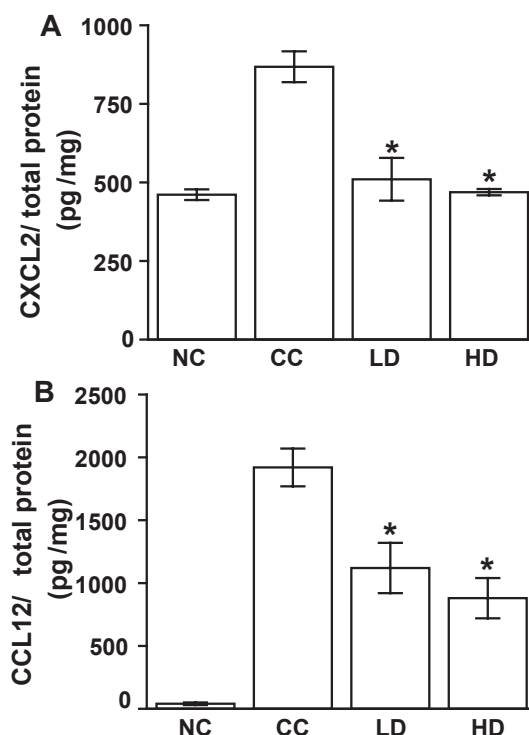


Fig. 4. Dietary pomegranate polyphenol concentrate (PPC) suppresses CXCL2 and CCL12 production in mice with contact hypersensitivity (CHS). Ear tissues were collected at 24 h after the second challenge with dinitrofluorobenzene, and chemokine levels were measured by enzyme-linked immunosorbent assay (ELISA) analysis. (A) CXCL2; (B) CCL12. NC, non-CHS control group; CC, CHS control group; LD, low-dose PPC treated group; HD, high-dose PPC-treated group. The data represent the results of two independent experiments [$n = 6$ mice per group, except for the non-CHS group ($n = 3$)] and are presented as means $\pm$ standard errors of the mean (SEM); $P < 0.05$ vs. CC.

ONO-AE-248 during the CHS elicitation phase resulted in significantly reduced CXCL1 and CXCL2 expression and inhibition of CHS. Lastly, Nakamura et al. (2016) studied the effects of glycyrrhizinic acid (GA), a traditional herbal medicine, on trichophytin-induced CHS. GA suppressed ear swelling and inhibited the expression of inflammatory cytokines, including CXCL2, IL-6, TNF- α , and IFN- γ . Administration of an anti-CXCL2 antibody also suppressed trichophytin-induced CHS. These results suggest that PPC downregulates the expression of CXCL2 and CCL12, thereby attenuating the infiltration of neutrophils and monocytes into allergic inflamed ear tissues.

Polyphenol-rich pomegranate fruit extract (POMx), punicalagin, and UA have anti-inflammatory properties. POMx decreased stimulated pro-inflammatory gene expression and the production of IL-6 and IL-8 in the human basophilic KU812 cell line. POMx also suppressed the induction of NF- κ B promoter-mediated luciferase activity in transiently transfected KU812 cells (Rasheed et al., 2009). The effects of EA, UA, and urolithin B (UB) were studied in colon fibroblasts upon IL-1 β treatment as an *in vitro* inflammation model. UA and UB, but not EA, attenuated the NF- κ B and mitogen-activated protein kinase pathways (González-Sarrías, Larrosa, Tomás-Barberán, Dolara, & Espín, 2010). These results suggest that urolithins, but not EA, are responsible for the anti-inflammatory properties of pomegranate fruit extract. The present study demonstrated that UA reduced INF- γ -producing splenocytes. INF- γ is a pro-inflammatory cytokine with antiviral activity, and high levels of INF- γ were observed at the elicitation sites of CHS (Grabbe & Schwarz, 1998). Mori et al. (2008) also reported that INF- γ acted on keratinocytes to produce chemokines, which lead inflammatory cell infiltration into the skin during the elicitation phase of CHS (Mori et al., 2008). We believe that this effect may be attributed to the anti-

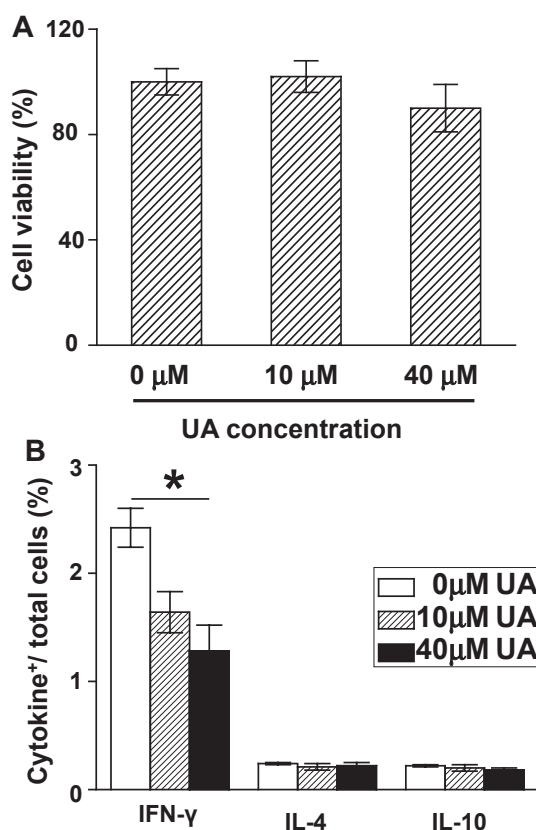


Fig. 5. Urolithin A (UA) reduces INF- γ -producing splenocytes in mice with contact hypersensitivity (CHS). UA was added at different concentrations to splenocytes of the CHS control mice, and the cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of monensin for 5 h. Cell viability was assessed with a trypan blue exclusion assay. The splenic cells were stained for the respective intracellular cytokine (interferon-gamma [IFN- γ], interleukin- [IL] 4, or IL-10). The stained cells were analyzed by FACS. (A) Cell viability. The values are expressed as the percentage of splenic cells treated without UA. (B) Cytokine-producing cells as the percentage of the total number of cells. Each value represents the mean $\pm$ SEM (n = 6); *p < 0.05 vs. 0 μ M UA.

inflammatory properties of UA.

5. Conclusions

Our data provide evidence that low-dose PPC diets are capable of attenuating CHS by inhibiting CXCL2 and CCL12 expression at CHS elicitation sites. These results also suggest that consumption of physiologically relevant doses of PPC may be of therapeutic value for ACD patients. Further research will be directed toward testing the beneficial effects of PPC on ACD in human subjects.

Acknowledgments

The authors thank N. Nishida (Morishita Jintan Co.) for kindly providing PPC. This work was supported by the Japan Ministry of Agriculture, Forestry and Fisheries, and JSPS KAKENHI (Grant Number 25350112).

Conflict of interests

The authors declare no conflict of interest.

References

- Aviram, M., & Rosenblat, M. (2012). Pomegranate protection against cardiovascular diseases. *Evidence-Based Complementary and Alternative Medicine*, 2012, 382763. <http://dx.doi.org/10.1155/2012/382763>.
- Bakkalbasi, E., Menteş, Ö., & Artik, N. (2008). Food ellagitannins—occurrence, effects of processing and storage. *Critical Reviews in Food Science and Nutrition*, 49(3), 283–298. <http://dx.doi.org/10.1080/10408390802064404>.
- Espin, J. C., Larrosa, M., Garcia-Conesa, M. T., & Tomas-Barberan, F. (2013). Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: The evidence so far. *Evidence-Based Complementary and Alternative Medicine*, 2013, 270418. <http://dx.doi.org/10.1155/2013/270418>.
- Faria, A., & Calhau, C. (2011). The bioactivity of pomegranate: Impact on health and disease. *Critical Reviews in Food Science and Nutrition*, 51(7), 626–634. <http://dx.doi.org/10.1080/10408391003748100>.
- Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry*, 48(10), 4581–4589. <http://dx.doi.org/10.1021/jf000404a>.
- González-Sarriás, A., Larrosa, M., Tomás-Barberán, F. A., Dolara, P., & Espín, J. C. (2010). NF- κ B-dependent anti-inflammatory activity of urolithins, gut microbiota ellagic acid-derived metabolites, in human colonic fibroblasts. *British Journal of Nutrition*, 104(4), 503–512. <http://dx.doi.org/10.1017/S0007114510000826>.
- Grabbe, S., & Schwarz, T. (1998). Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunology Today*, 19(1), 37–44. [http://dx.doi.org/10.1016/S0167-5699\(97\)01186-9](http://dx.doi.org/10.1016/S0167-5699(97)01186-9).
- Grabbe, S., Steinert, M., Mahnke, K., Schwartz, A., Luger, T. A., & Schwarz, T. (1996). Dissection of antigenic and irritative effects of epicutaneously applied haptens in mice. *Journal of Clinical Investigation*, 98(5), 1158–1164.
- Heber, D. (2008). Multitargeted therapy of cancer by ellagitannins. *Cancer Letters*, 269(2), 262–268. <http://dx.doi.org/10.1016/j.canlet.2008.03.043>.
- Honda, T., Egawa, G., Grabbe, S., & Kabashima, K. (2013). Update of immune events in the murine contact hypersensitivity model: Toward the understanding of allergic contact dermatitis. *Journal of Investigative Dermatology*, 133(2), 303–315. <http://dx.doi.org/10.1038/jid.2012.284>.
- Honda, T., Matsuoka, T., Ueta, M., Kabashima, K., Miyachi, Y., & Narumiya, S. (2009). Prostaglandin E2-EP3 signaling suppresses skin inflammation in murine contact hypersensitivity. *Journal of Allergy and Clinical Immunology*, 124(4), 809–818. <http://dx.doi.org/10.1016/j.jaci.2009.04.029>.
- Ito, H., Li, P., Koreishi, M., Nagatomo, A., Nishida, N., & Yoshida, T. (2014). Ellagitannin oligomers and a neolignan from pomegranate arils and their inhibitory effects on the formation of advanced glycation end products. *Food Chemistry*, 152, 323–330. <http://dx.doi.org/10.1016/j.foodchem.2013.11.160>.
- Kaplan, D. H., Igyártó, B. Z., & Gaspari, A. A. (2012). Early events in the induction of allergic contact dermatitis. *Nature Reviews Immunology*, 12(2), 114–124. <http://dx.doi.org/10.1038/nri3150>.
- Kawakami, K., Li, P., Uraji, M., Hatanaka, T., & Ito, H. (2014). Inhibitory effects of pomegranate extracts on recombinant human maltase-glucoamylase. *Journal of Food Science*, 79(9), H1848–H1853. <http://dx.doi.org/10.1111/1750-3841.12568>.
- Larrosa, M., Garcia-Conesa, M. T., Espin, J. C., & Tomas-Barberan, F. A. (2010). Ellagitannins, ellagic acid and vascular health. *Molecular Aspects of Medicine*, 31(6), 513–539. <http://dx.doi.org/10.1016/j.mam.2010.09.005>.
- Martin, S. F., Esser, P. R., Weber, F. C., Jakob, T., Freudenberg, M. A., Schmidt, M., & Goebeler, M. (2011). Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy*, 66(9), 1152–1163. <http://dx.doi.org/10.1111/j.1398-9995.2011.02652.x>.
- Mitsui, G., Hirano, T., Niwano, Y., Mitsui, K., Ohara, O., Yanagihara, S., & Kato, M. (2004). Effect of a topical steroid on gene expressions for chemokines in mice with contact hypersensitivity. *International Immunopharmacology*, 4(1), 57–69. <http://dx.doi.org/10.1016/j.intimp.2003.10.005>.
- Mitsui, G., Mitsui, K., Hirano, T., Ohara, O., Kato, M., & Niwano, Y. (2003). Kinetic profiles of sequential gene expressions for chemokines in mice with contact hypersensitivity. *Immunology Letters*, 86(2), 191–197. [http://dx.doi.org/10.1016/S0165-2478\(03\)00017-8](http://dx.doi.org/10.1016/S0165-2478(03)00017-8).
- Mori, T., Kabashima, K., Yoshiki, R., Sugita, K., Shiraishi, N., Onoue, A., & Tokura, Y. (2008). Cutaneous hypersensitivities to hapten are controlled by IFN- γ -upregulated keratinocyte Th1 chemokines and IFN- γ -downregulated langerhans cell Th2 chemokines. *Journal of Investigative Dermatology*, 128(7), 1719–1727. <http://dx.doi.org/10.1038/jid.2008.5>.
- Nagano, T., Nishida, N., & Ito, H. (2018). The inhibitory effect of a polyphenol concentrate from pomegranate juice on 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice. *Food Science and Technology Research*, 24(1), 169–175. <http://dx.doi.org/10.3136/fstr.24.169>.
- Nagano, T., Wu, W., Tsumura, K., Yonemoto-Yano, H., Kamada, T., & Haruma, K. (2016). The inhibitory effect of soybean and soybean isoflavone diets on 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice. *Bioscience, Biotechnology, and Biochemistry*, 80(5), 991–997. <http://dx.doi.org/10.1080/09168451.2015.1132150>.
- Nakamura, T., Nishibu, A., Yoshida, N., Yasoshima, M., Anzawa, K., Watanabe, Y., & Mochizuki, T. (2016). Glycyrrhetic acid inhibits contact hypersensitivity induced by trichophyton via dectin-1. *Experimental Dermatology*, 25(4), 299–304. <http://dx.doi.org/10.1111/exd.12931>.
- Niwano, Y., Mitsui, G., & Kohno, M. (2008). Chemokines and their receptors as a target for the treatment of contact hypersensitivity. *Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry*, 7(1), 45–51. <http://dx.doi.org/10.2174/>

- 187152308783769159.
- Rasheed, Z., Akhtar, N., Anbazhagan, A. N., Ramamurthy, S., Shukla, M., & Haqqi, T. M. (2009). Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF- κ B in human KU812 cells. *Journal of Inflammation*, 6(1), 1. <http://dx.doi.org/10.1186/1476-9255-6-1>.
- Shornick, L. P., De Togni, P., Mariathasan, S., Goellner, J., Strauss-Schoenberger, J., Karr, R. W., & Chaplin, D. D. (1996). Mice deficient in IL-1 β manifest impaired contact hypersensitivity to trinitrochlorobenzene. *The Journal of Experimental Medicine*, 183(4), 1427–1436. <http://dx.doi.org/10.1084/jem.183.4.1427>.
- Sutterwala, F. S., Ogura, Y., Szczepanik, M., Lara-Tejero, M., Lichtenberger, G. S., Grant, E. P., & Flavell, R. A. (2006). Critical role for NALP3/CIA1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity*, 24(3), 317–327. <http://dx.doi.org/10.1016/j.immuni.2006.02.004>.
- Wakabayashi, T., Hu, D. L., Tagawa, Y., Sekikawa, K., Iwakura, Y., Hanada, K., & Nakane, A. (2005). IFN- γ and TNF- α are involved in urushiol-induced contact hypersensitivity in mice. *Immunology & Cell Biology*, 83(1), 18–24. <http://dx.doi.org/10.1111/j.1440-1711.2005.01310.x>.
- Watanabe, H., Gaide, O., Petrilli, V., Martinon, F., Contassot, E., Roques, S., & French, L. E. (2007). Activation of the IL- β -processing inflammasome is involved in contact hypersensitivity. *Journal of Investigative Dermatology*, 127(8), 1956–1963. <http://dx.doi.org/10.1038/sj.jid.5700819>.