



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The isoflavone puerarin induces Foxp3⁺ regulatory T cells by augmenting retinoic acid production, thereby inducing mucosal immune tolerance in a murine food allergy model

Takeshi Yamamoto*, Emi Matsunami, Koji Komori, Shusaku Hayashi, Makoto Kadowaki

Division of Gastrointestinal Pathophysiology, Institute of Natural Medicine, University of Toyama, Toyama, Japan

ARTICLE INFO

Article history:

Received 1 June 2019

Accepted 9 June 2019

Available online xxx

Keywords:

Puerarin

Regulatory T cells

Retinoic acid

Aldh1a1

Food allergy

Mucosal immune tolerance

ABSTRACT

The disruption of intestinal mucosal immune tolerance can lead to the development of intestinal immune diseases such as food allergy (FA). Regulatory T cells (Tregs) in the mucosa play a critical role in maintaining peripheral immune tolerance in the intestine, and retinoic acid (RA) is absolutely required for the induction of Tregs. We have previously reported that kakkonto, a traditional Japanese herbal medicine, suppresses FA in a murine FA model due to the induction of Tregs in the colonic mucosa. However, the precise molecular mechanisms underlying the induction of Tregs remain unclear. Puerarin, an isoflavone derivative, is a major constituent of kakkonto. Thus, we investigated the effect of puerarin on the induction of Tregs. BALB/c mice were systemically sensitized and then orally challenged with ovalbumin (OVA) as an FA model. Puerarin treatment suppressed the development of allergic diarrhea in FA mice. The gene expression levels of IL-4 and mast cell protease 1 (mMCP-1) were significantly upregulated in the proximal colon of FA mice but were reduced by puerarin. The proportions of Foxp3⁺CD4⁺ cells and CD103⁺CD11c⁺ dendritic cells (DCs) were significantly higher among the colonic lamina propria (cLP) cells of puerarin-treated FA mice than among those of untreated FA mice. The gene expression of Aldh1a1, an RA synthesis enzyme, in colonic epithelial cells (CECs) was significantly higher in the puerarin-treated FA mouse colon than in the untreated FA mouse colon. In addition, the preventive effect of puerarin was suppressed in the FA model by pretreatment with LE540, an RA receptor (RAR) antagonist. The induction of Foxp3⁺CD4⁺ cells and CD103⁺CD11c⁺ DCs by puerarin was reduced by pretreatment with LE540. The present findings indicate that the augmentation of RA production in CECs induced by puerarin enhances the induction of Tregs and suppresses the development of FA in a mouse model. Thus, a natural enhancer of RA production, such as puerarin, has the potential to treat immune diseases attributed to Treg deficiency.

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1. Introduction

Food allergy (FA) is an abnormal immune reaction due to non-tolerance to some specific foods. In recent decades, FA has become increasingly prevalent, especially in developed countries [1]. Currently, there is no approved long-term effective medicine for FA. Therefore, the treatments for FA patients are the avoidance of FA-triggering foods and the management of allergic hypersensitivities known as anaphylaxis.

The development of FA is attributed to a disturbance in oral

tolerance induction in the intestinal mucosal immune system [2]. Foxp3⁺ regulatory T cells (Tregs) play a key role in the establishment of oral tolerance. Hadis et al. reported that FA responses are enhanced by the depletion of Foxp3⁺ Tregs and that oral tolerance is induced by the expansion of intestinal Foxp3⁺ Tregs [3]. Wang et al. reported that the intake of a low dose of antigen promotes the induction of Foxp3⁺ Tregs in the jejunum and mesenteric lymph node (MLN), thereby suppressing allergic responses [4]. However, it remains unclear how Foxp3⁺ Tregs expand in the intestine.

We have previously reported that kakkonto, a traditional Japanese herbal medicine, is a potential suppressive drug that prevents the development of FA in our FA mouse model [5] and that kakkonto induces Foxp3⁺ Tregs in the colonic lamina propria (cLP) cell population [6]. Puerarin is an isoflavonoid isolated from the root of

* Corresponding author.

E-mail address: ty@inm.u-toyama.ac.jp (T. Yamamoto).

Pueraria lobata and one of the main bioactive components of kak-konto. Puerarin possesses many pharmacological activities [7]. Puerarin exerts antiallergic effects on several animal models. Puerarin suppresses the expression of Th2 cytokines and inhibits the recruitment of eosinophils into the lungs of ovalbumin (OVA)-induced asthma mice [8]. However, little is known about the effect of puerarin on intestinal mucosal immune responses.

Retinoic acid (RA), the active derivative of Vitamin A (VA), plays an important role in immune responses [9]. In particular, the differentiation of Foxp3⁺ Tregs [10] and tolerogenic CD103⁺ dendritic cells (DCs) [11] depends on the amount of RA. Humans are not able to synthesize VA and thus depend on dietary replenishment. In the intestine, RA is synthesized from VA via the enzymatic reactions of retinaldehyde dehydrogenase 1a1 (Aldh1a1). Aldh1a1 is expressed at high levels in intestinal epithelial cells and maintains RA levels in the intestinal mucosa of humans [12] and mice [13]. Thus, the expression of Aldh1a1 in intestinal epithelial cells contributes to immune tolerance in the intestine by regulating intestinal RA levels.

In this study, we investigated the effect of puerarin on FA model mice and the underlying mechanisms, especially those involved in the induction of immune tolerance in the intestine.

2. Materials and methods

2.1. Animals

Male BALB/c mice (4–8 weeks old) and Rag2^{-/-} DO11.10 TCR transgenic mice were purchased from SLC (Shizuoka, JAPAN) and Taconic Laboratories (Germantown, NY, USA). This study was approved by the Animal Experiment Committee at the University of Toyama (authorization No. A2018 INM-4 and A2015 INM-3).

2.2. FA model and drug treatment

FA induction was performed as previously described [5,6,14]. Briefly, BALB/c mice were sensitized twice at two-week intervals by intraperitoneal (ip) injection of 50 µg of OVA in the presence of an aluminum hydroxide gel adjuvant. Two weeks later, the mice were repeatedly given 50 mg of OVA 3 times per week. Diarrhea was assessed by visually monitoring the mice for up to 1 h following the oral OVA challenge. Puerarin (1, 5, 25, or 50 mg/kg) was orally administered to mice 1 h before oral OVA challenge. LE540 (2.5 mg/kg; Wako, Osaka, Japan) was intraperitoneally administered to mice 2 h before puerarin administration.

2.3. Cell isolation of cLP cells, colonic epithelial cells (CECs) and splenocytes

cLP cells were isolated from the colon as previously described [6,14]. Briefly, excised colons were incubated at 37 °C in 2% fetal bovine serum (FBS) RPMI 1640 medium containing 0.05 mM EDTA for 20 min. cLP cells were isolated using an enzymatic dissociation procedure with collagenase (for cell dispersion; Wako) and then the Percoll density gradient centrifugation method. CECs were isolated from the cells separated by 0.05 mM EDTA. Splenocytes were isolated by an enzymatic dissociation procedure with collagenase III (Worthington Biochemical Corp., Freehold, NJ, USA).

2.4. Cell staining and flow cytometry analysis

To analyze cell surface markers by flow cytometry, isolated cells were stained with the following monoclonal antibodies (mAbs): FITC-conjugated anti-mouse CD3ε (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-mouse CD4 (Miltenyi Biotec),

FITC-conjugated anti-mouse CD103 (BioLegend, San Diego, CA, USA), and APC-conjugated anti-mouse CD11c (BD Biosciences, San Jose, CA, USA). For intracellular staining, cells were permeabilized with the Foxp3 Staining Buffer Set (eBioscience, San Diego, USA) following the manufacturer's instructions. Thereafter, the cells were stained with an APC-conjugated anti-mouse/rat Foxp3 antibody (eBioscience). Flow cytometry analysis was performed using a FACS Canto II (BD Biosciences).

2.5. Quantitative real-time PCR

Total RNA was isolated from colonic tissue and CECs by Sepasol Super (Nacalai Tesque). An ExScript RT reagent kit (Takara Bio, Shiga, Japan) and PCR Thermal Cycler Dice (Takara Bio) were used for RNA reverse transcription. Quantitative real-time PCR was then performed using SYBR Premix Ex Taq (Takara Bio) and a Takara TP800 Real-time PCR system (Takara Bio).

The following primer pairs were used: interleukin (IL)-4, forward 5'-GGTCTCAACCCAGCTAGT-3' and reverse 5'-GCCGATGATCTCTCAAGTGAT-3'; mast cell protease 1 (mMCP-1), forward 5'-CCATCTGAAGATCATCACGGACA-3' and reverse 5'-ACATCATGAGCTCCAAGGTGAC-3'; Aldh1a1, forward 5'-GGATTCAGATGTCTGGAAATGGA-3' and reverse 5'-TGCGACTGCTTGAGCTCAGTGTA-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-TGACCA-CAGTCCATGCCATC-3' and reverse 5'-GACGGACACATTGGGGGTAG-3'.

The levels of target mRNA expression were normalized to the GAPDH mRNA level as an internal control for each sample.

2.6. In vitro Treg differentiation assay

Treg differentiation assays were performed according to a method described previously [14]. Briefly, naïve CD4⁺ T cells were separated from the splenocytes of Rag2^{-/-} DO11.10 TCR transgenic mice and labeled with carboxyfluorescein succinimidyl ester (CFSE). The CFSE-labeled naïve CD4⁺ T cells (2.0 × 10⁵ cells/well) were cultured with DCs (2.0 × 10⁴ cells/well) isolated from the splenocytes of normal mice in the presence of OVA peptide 323–339, an anti-interferon (IFN)-γ mAb, an anti-IL-4 mAb, recombinant mouse IL-2, recombinant human transforming growth factor (TGF)-β1 and/or puerarin for 5 days. The CFSE-labeled CD4⁺ T cells were assessed using flow cytometry.

2.7. In vitro experiments using bone marrow-derived dendritic cells (BMDCs)

BMDCs were prepared from the femurs and tibias of BALB/c mice according to a method described previously [15]. Briefly, immature BMDCs were obtained using GM-CSF (10 ng/mL; R&D Systems, Minnesota, USA). After treatment with puerarin for 24 h, immature BMDCs were incubated with FITC-OVA (10 µg/mL; Invitrogen) for 60 min at 37 °C, and fluorescence intensity was assessed by flow cytometry. After treatment with puerarin for 24 h, immature BMDCs were cultured with lipopolysaccharide (LPS). Adherent mature BMDCs were stained with mAbs against CD11c, MHC class II, CD80, CD86 and CD103 and analyzed by flow cytometry. Immature BMDCs were incubated with 100 ng/ml LPS for 24 h at 37 °C in RA-containing medium (1–1000 nM). Adherent mature BMDCs were stained with mAbs against CD11c and CD103 and analyzed by flow cytometry.

2.8. Statistical analyses

The data are expressed as the mean ± SE. Statistical comparisons

were made with an unpaired Student *t*-test, the χ^2 test or one-way ANOVA followed by Dunnett's test or the Bonferroni/Dunn tests for multiple comparisons. Probability (*P*) values < 0.05 were considered statistically significant.

3. Results and discussion

The extract of the root of *Pueraria lobata*, one of the constituent herbal medicines of kakkonto, significantly suppressed the occurrence of allergic diarrhea in FA mice (Supplementary Fig. 1). Puerarin is a major constituent of the root of *Pueraria lobata* and kakkonto.

Therefore, to investigate whether puerarin can suppress the development of FA, puerarin was administered 1 h before each oral OVA challenge. As shown in Fig. 1A, allergic diarrhea began to occur approximately 30 min after the 3rd oral OVA challenge. The occurrence of allergic diarrhea increased in proportion to the number of oral OVA challenges and reached $77.5 \pm 7.7\%$ after the 6th OVA challenge. Puerarin significantly reduced the occurrence of allergic diarrhea to $45.9 \pm 10.7\%$ after the 6th OVA challenge (*P* < 0.05). Furthermore, puerarin suppressed the occurrence of allergic diarrhea in a dose-dependent manner (Fig. 1B, *P* < 0.01–0.05).

The intestinal mucosal immune system is more important than the systemic immune system in the development of FA by showing that splenectomy fails to prevent allergic diarrhea [16]. Enhanced expression of Th2 cytokines (IL-4 and IL-13) in the intestine is concomitant with the development of FA symptoms [5,6,17–19].

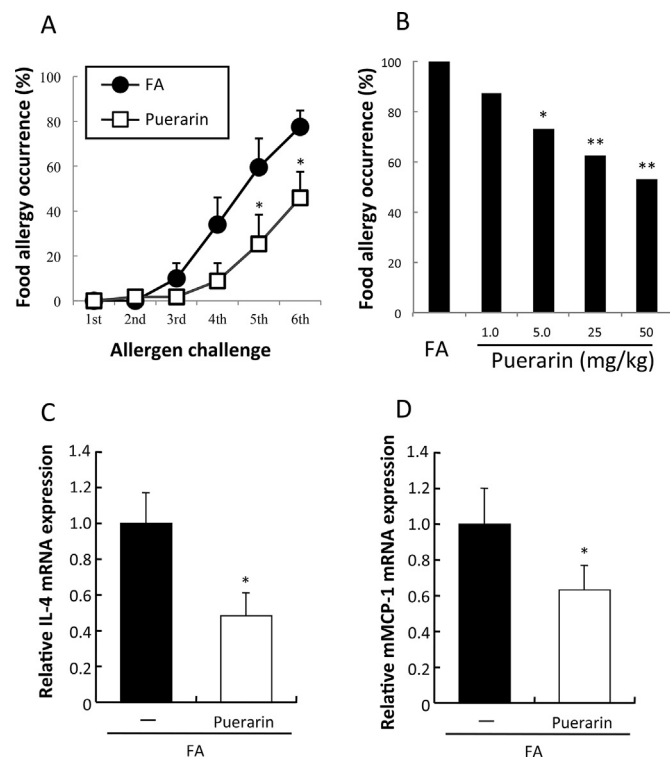


Fig. 1. Suppressive effect of puerarin on the occurrence of allergic diarrhea. A: The occurrence of allergic diarrhea in untreated FA mice (●) and puerarin-treated FA mice (□; 25 mg/kg, po) is shown. The data were obtained from 5 independent experiments (total *n* = 45). **P* < 0.05 vs. the untreated FA mice. B: Puerarin suppressed the occurrence of allergic diarrhea in a dose-dependent manner (*n* = 8–21 mice per group). **P* < 0.05 and ***P* < 0.01 vs. the FA mice. C, D: The mRNA expression levels of IL-4 (C) and mMCP-1 (D) in the proximal colon of untreated FA mice and puerarin-treated FA mice were measured by real-time PCR. **P* < 0.05 vs. the untreated FA mice (*n* = 4–9 mice per group).

Therefore, we evaluated the effect of puerarin on the mRNA expression of the Th2 cytokine IL-4 in the proximal colon by real-time PCR. The mRNA expression of IL-4 was significantly down-regulated in puerarin-treated FA mice (Fig. 1C, *P* < 0.05). Next, we assessed the infiltration of mucosal-type mast cells. We previously reported that the development of FA symptoms depends upon the hyperplasia and degranulation of mucosal-type mast cells in the colon [5,6,17]. A previous clinical report demonstrated that the level of mast cell tryptase, a mast cell-associated marker, is elevated in gut mucosa biopsy samples from FA patients with gastrointestinal symptoms [20]. Puerarin significantly reduced the mRNA expression of mMCP-1 in puerarin-treated FA mice (Fig. 1D, *P* < 0.05).

The defective generation of functional Tregs caused by mutations in the human *Foxp3* gene results in a variety of immune diseases, including severe FA [21]. Similarly, *Foxp3* mutant mice also exhibit allergic dysregulation [22]. In addition, an adoptive transfer of in vitro-differentiated CD4⁺CD25⁺*Foxp3*⁺ Tregs ameliorates peanut allergy in a murine model [23]. Therefore, these previous reports indicate that inducers of abundant and functional Tregs in the intestine have the potential to suppress the development of FA. Thus, we examined the proportion of Tregs in the cLP cell population. CD4⁺*Foxp3*⁺ Tregs were assessed by flow cytometry after the 6th OVA administration. The proportion of CD4⁺*Foxp3*⁺ Tregs among the CD4⁺ T cells in the cLP cell population of puerarin-treated FA mice ($26.3 \pm 2.2\%$) was higher than that in untreated FA mice ($20.0 \pm 1.4\%$) (Fig. 2A, *P* < 0.01).

To investigate the mechanism underlying the induction of CD4⁺*Foxp3*⁺ Tregs by puerarin in FA mice, we next assessed the direct effect of puerarin on the differentiation of CD4⁺*Foxp3*⁺ Tregs. However, puerarin had no effect on the proportion of CD4⁺*Foxp3*⁺ Tregs in an in vitro Treg differentiation assay (Fig. 2B).

We previously reported that RA significantly augments the differentiation of CD4⁺*Foxp3*⁺ Tregs from naïve CD4⁺ T cells in a dose-dependent manner [14]. *Foxp3*⁺ Tregs are differentiated from naïve CD4⁺ T cells by the combination of RA and TGF- β [14,24]. In the intestine, the RA synthase *Aldh1a1* is highly expressed in intestinal epithelial cells [12,13]. Therefore, we investigated whether RA is involved in the suppressive effect of puerarin on the occurrence of allergic diarrhea in FA mice. Puerarin treatment significantly upregulated the mRNA expression level of *Aldh1a1* in the CECs of FA mice (Fig. 3A, *P* < 0.01). In addition, the RA receptor (RAR) antagonist LE540 was used to suppress the activation of the RA signaling pathway. Pretreatment with LE540 inhibited the suppressive effect of puerarin (25 mg/kg po) on the occurrence of allergic diarrhea (Fig. 3B, *P* < 0.01). Pretreatment with LE540 completely suppressed the increased proportion of CD4⁺*Foxp3*⁺ T cells in the cLP cell population of puerarin-treated FA mice (Fig. 3C, *P* < 0.01). We have also reported that kakkonto treatment does not affect the mRNA expression of TGF- β in the colon by using microarray analysis [5]. Taken together, these findings suggested that puerarin increases the RA concentration in the colon by enhancing RA production in CECs and thereby induce CD4⁺*Foxp3*⁺ Tregs in the cLP cell population.

Intestinal CD103⁺ DCs can exert tolerogenic effects by expressing *Aldh1a2* and producing RA and drive the differentiation of intestinal *Foxp3*⁺ Tregs via a TGF- β and RA-dependent mechanism [25]. Conversely, RA reportedly increases *Aldh1a2* mRNA expression in BMDCs [26]. We examined whether puerarin induces tolerogenic DCs in the colon of FA mice. The proportion of CD103⁺CD11c⁺ DCs in the cLP cell population was significantly increased in puerarin-treated FA mice ($0.6 \pm 0.1\%$) compared to untreated FA mice ($0.3 \pm 0.1\%$) (Fig. 4A, *P* < 0.05). However, puerarin did not influence FITC-OVA uptake by BMDCs (Fig. 4B) nor the surface expression of CD80, CD86, MHC class II and CD103 on

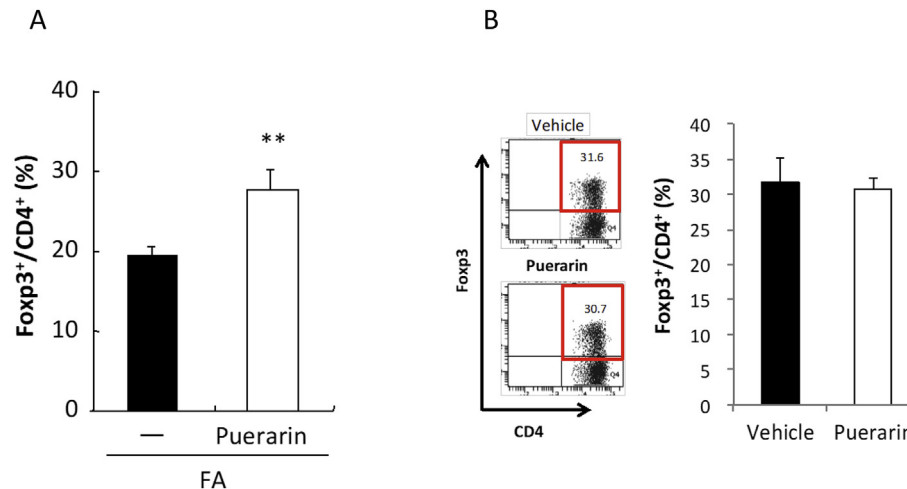


Fig. 2. Effect of puerarin on the proportion of Tregs in the colon of FA mice and the differentiation of CD4⁺Foxp3⁺ Tregs in vitro. A: The proportion of CD4⁺Foxp3⁺ Tregs among the CD4⁺ T cells in the cLP cell population of untreated FA mice and puerarin-treated FA mice was analyzed by flow cytometry. **P* < 0.05 and ***P* < 0.01 vs. the untreated FA mice (*n* = 6–7 mice per group). B: Representative dot plots and the proportions of CD4⁺Foxp3⁺ Tregs in vehicle-containing medium and puerarin-containing medium are shown. Naïve CD4⁺ T cells and DCs from the spleen were cocultured in the presence or absence of 10 μM puerarin (*n* = 4–5 per group).

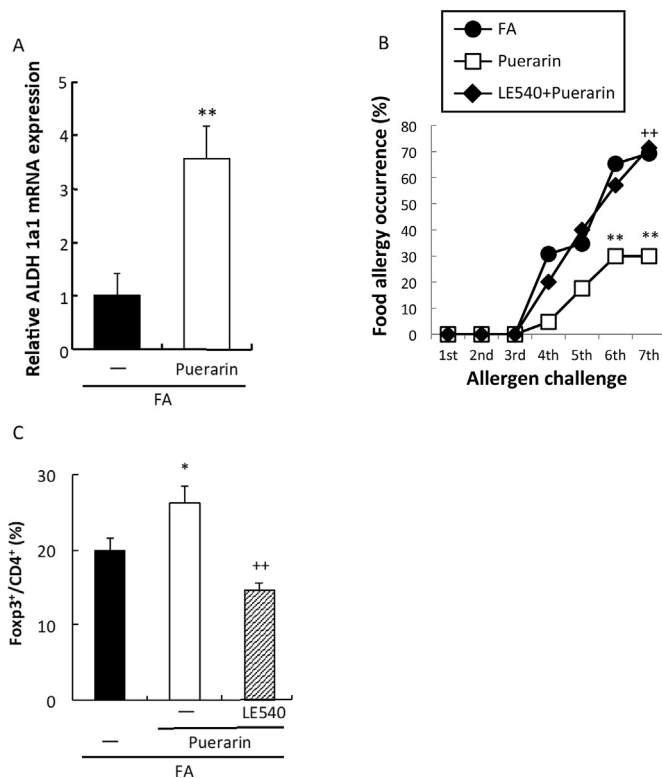


Fig. 3. Involvement of the RA signaling pathway in the suppressive effect of puerarin on the occurrence of allergic diarrhea in FA mice. A: The mRNA expression of Aldh1a1 in the CECs of untreated FA mice and puerarin-treated FA mice was measured by real-time PCR. **P* < 0.05 vs. the untreated FA mice (*n* = 8 mice per group). B: The occurrence of allergic diarrhea in untreated FA mice (●), puerarin-treated FA mice (□; 25 mg/kg, po) and LE540-pretreated puerarin-treated FA mice (◆; puerarin 25 mg/kg, po; LE540 2.5 mg/kg, ip) is shown. ***P* < 0.01 vs. the untreated FA mice, ++*P* < 0.01 vs. the puerarin-treated FA mice. (*n* = 14–40 per group). C: The proportion of CD4⁺Foxp3⁺ Tregs among the CD4⁺ T cells in the cLP cell population in untreated FA mice, puerarin-treated FA mice and LE540-pretreated puerarin-treated FA mice was analyzed by flow cytometry. **P* < 0.05 vs. the untreated FA mice, ++*P* < 0.01 vs. the puerarin-treated FA mice (*n* = 4–11 mice per group).

BMDCs (Fig. 4C) in an in vitro assay.

Several recent studies have indicated that the induction of tolerogenic intestinal CD103⁺ DCs is promoted by intestinal epithelial cells through RA production. Human monocyte-derived DCs become tolerogenic after incubating with human intestinal epithelial cells, and then these DCs are able to induce Foxp3⁺ Treg differentiation [27]. In a murine experiment, Aldh1a1-expressing epithelial cells are shown to induce tolerogenic CD103⁺ DCs through RA production, and these tolerogenic CD103⁺ DCs can induce Foxp3⁺ Tregs [28]. In the present study, RA upregulated the proportion of CD103⁺CD11c⁺ DCs in a BMDC population in a dose-dependent manner (Fig. 4D, *P* < 0.01–0.05). Puerarin increased Aldh1a1 expression in CECs, and pretreatment with LE540 reduced the increased proportion of CD103⁺ DCs in the cLP cell population of puerarin-treated FA mice (Fig. 4E).

These findings indicate that RA produced in CECs exerts a tolerogenic function in the intestine and that the expression of Aldh1a1 in CECs correlates with the levels of RA in the colonic mucosa. The conclusion regarding RA is supported by clinical reports showing that Aldh1a1 expression is lower in patients with Crohn's disease than in normal subjects [12] and that the proportion of CD103⁺ DCs is lower in the intestinal mucosa of Crohn's disease patients than in that of normal subjects [29]. Collectively, our data support the notion that the Aldh1a1 expression in CECs upregulated by puerarin enhances the production of RA, and subsequently, a high level of RA leads to the induction of tolerogenic CD103⁺ DCs and the concomitant induction of Foxp3⁺ Tregs by RA released from CD103⁺ DCs.

Therefore, it is assumed that a sufficient level of RA maintains immune tolerance in the intestine. However, an excess of RA has adverse effects, such as abnormal fetal development and disruptions in the regulation of the energy balance [30]. In contrast, puerarin has few adverse effects since the RA level is increased only in the colonic tissue by inducing Aldh1a1 expression in CECs.

In conclusion, puerarin is able to induce Aldh1a1 expression in CECs, and then the enhanced RA production leads to the induction of tolerogenic CD103⁺ DCs and Foxp3⁺ Tregs in the cLP cell population. Consequently, a natural enhancer of RA production, such as puerarin, has the potential to maintain immune tolerance and thereby suppress the development of FA.

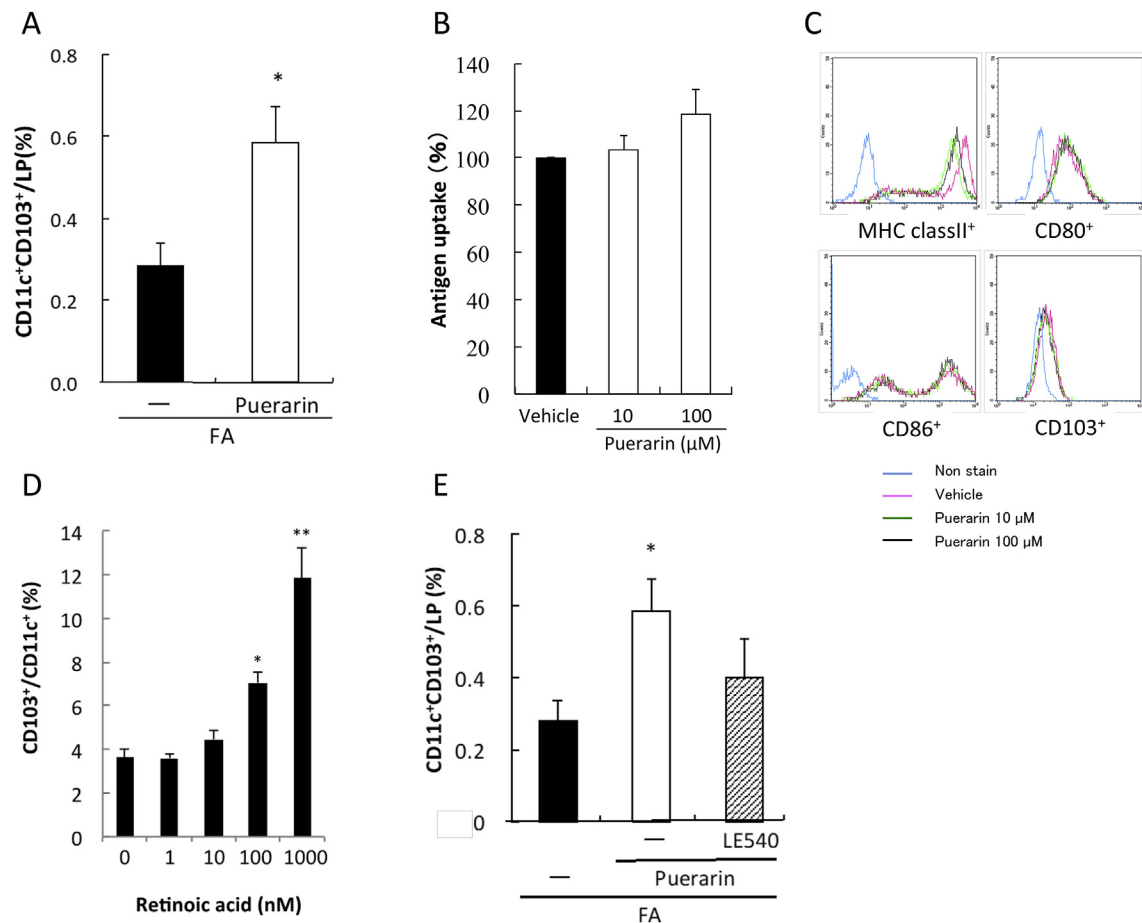


Fig. 4. Effect of puerarin on the induction of tolerogenic DCs in the colon of FA mice. A: The effect of puerarin on the proportion of tolerogenic DCs in the cLP cell population of FA mice. The proportion of CD103⁺CD11c⁺ DCs in the cLP cell population of untreated FA mice and puerarin-treated FA mice was analyzed by flow cytometry. **P* < 0.05, ***P* < 0.01 vs. the untreated FA mice (*n* = 6–7 mice per group). B: The rates of BMDC uptake of FITC-OVA in vehicle-containing medium and puerarin-containing medium. The fluorescence intensity in BMDCs was assessed by flow cytometry. The data were calculated as a percent of the value obtained for the vehicle-containing medium and are expressed as the mean ± SE obtained from 5 independent experiments. C: Histogram showing the representative expression of MHC class II, CD80, CD86 and CD103 in BMDCs. Fluorescence intensity was assessed by flow cytometry. D: Dose-dependent effect of RA on the differentiation of BMDCs into CD103⁺CD11c⁺ DCs in vitro. The data are expressed as the mean ± SE obtained from 3 independent experiments. **P* < 0.05 and ***P* < 0.01 vs. vehicle. E: The proportion of CD103⁺CD11c⁺ DCs in the cLP cell population of untreated FA mice, puerarin-treated FA mice and LE540-pretreated puerarin-treated FA mice (Fig. 3B) was analyzed by flow cytometry. **P* < 0.05 vs. the untreated FA mice (*n* = 4–11 mice per group).

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number JP16K09234 and JP19K07908.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.06.051>.

Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.06.051>.

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Figure S1

