



Oral administration with diosgenin enhances the induction of intestinal T helper 1-like regulatory T cells in a murine model of food allergy



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ABSTRACT

Although the development of T helper (Th)1-like regulatory T (Treg) cells under Th1 inflammatory conditions has been reported, the role of Th1-like Treg cells in Th2 allergic responses remains mostly unclear. We previously demonstrated that diosgenin, the major sapogenin contained in the Chinese yam, attenuated food allergy and augmented Th1 and Treg immune responses. In this study, we hypothesized that diosgenin may enhance the induction of Th1-like Treg cells in the gut of mice with food allergy. Ovalbumin (OVA)-sensitized BALB/c mice were gavaged daily with diosgenin and received repeatedly intragastric ovalbumin challenges to induce intestinal allergic responses. The induction of Foxp3⁺ Treg cells co-expressing Th1-type transcription factors, cytokines and chemokines in the intestine was examined, and the mRNA expression of the chemokines corresponding to Th1-like Treg cells was measured. Diosgenin administration increased the number of Foxp3⁺ Treg cells co-expressing Th1 markers, including CCR5, CXCR3, IFN- γ and T-bet in the intestine, and enhanced populations of Foxp3⁺IFN- γ ⁺ and Foxp3⁺T-bet⁺ cells that expressed the regulatory cytokine IL-10 in the Peyer's patches. Diosgenin also augmented the intestinal expression of CXCR3, CCL3, and CXCL10. Concordantly, diosgenin increased the number of CXCR3⁺Foxp3⁺IL-10 cells in the Peyer's patches. Our data demonstrated the enhanced induction of Th1-like Treg cells in allergic mice treated with diosgenin, providing evidence to suggest a role for Th1-like Treg cells in diosgenin-mediated anti-allergic effects against Th2-type allergy.

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1. Introduction

Food allergy, an atopic disorder to dietary proteins, affects up to 6% of the population in developed countries [1]. Symptoms of food allergy range from mild cutaneous and gastrointestinal illness to severe anaphylactic shock and even death. The pathophysiology of food allergy is characterized by a skewed Th2 polarization and an impaired regulatory immunity [1]. At present, limited therapeutic modality targeting the immunopathology of food allergy is available. Pharmacotherapy is primarily employed for symptom relief of hypersensitivity reactions. Avoidance of allergens is the major way to prevent the occurrence of

food allergy. Therefore, development of potential therapeutic and preventive measures for managing food allergy is of great importance.

Because the gastrointestinal tract encounters a myriad of dietary antigens, it has to be discreet for the gut immune system to determine whether an encountered antigen should be tolerated or reacted [2]. Regulatory T (Treg) cells play a pivotal role in maintaining the gut immunobalance [3–5]. Treg cells may release immunoregulatory cytokines, including interleukin (IL)-10 and transforming growth factor (TGF)- β to suppress immunocompetent cells, such as Th1 or Th2 effector cells [3–5]. Based on their ontogeny, conventional Treg cells can be classified as natural and induced Treg cells, both of which express the lineage-determining transcription factor forkhead box protein 3 (Foxp3) [6]. Although it has been considered that the differentiation of naïve Th cells to distinct effector lineages is irreversible, accumulating evidence suggest that induced Treg cells are more plastic and flexible than Th1 and Th2 cells [7]. For example, a group of unique Th1-like Treg subset expressing both Th1 and Treg markers can be induced under Th1-overreactive conditions, which plays an important role in controlling and ameliorating certain immune disorders, especially Th1 cell-mediated inflammation [8,9]. However, it remains mostly unclear

Abbreviations: CCL, chemokine (C-C motif) ligand; CCR, C-C chemokine receptor; CXCL, chemokine (C-X-C motif) ligand; CXCR, C-X-C chemokine receptor; Foxp3, forkhead box P3; IFN, interferon; Ig, immunoglobulin; IHC, immunohistochemical; IL, interleukin; OVA, ovalbumin; RT-PCR, reverse transcription-polymerase chain reaction; TGF, transforming growth factor; Th, T helper; Treg, regulatory T.

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if Th1-like Treg cells play a role in the pathophysiology of food allergy and the gut immunobalance.

The Chinese yam (*Dioscorea opposita* Thunb.), a superb herb in traditional Chinese medicine, has long been used to improve the immune and gastrointestinal functions. The Chinese yam contains a variety of phytochemicals, including amino acids, mucopolysaccharides, protein, sapogenins, saponins, etc. [10]. Diosgenin, the most abundant yam-derived sapogenin, has been suggested as one of the active ingredients contributing to many of yam-mediated effects, such as anti-inflammatory and immunomodulatory effects [11–13]. We previously reported that diosgenin enhanced the functionality of Th1 cells, as evidenced by an up-regulated expression of T-bet and antigen-induced interferon (IFN)- γ by spleen cells of ovalbumin (OVA)-sensitized mice [11,13]. Concordantly, diosgenin elevated the serum level of antigen-specific IgG_{2a}, but inhibited the production of total- and antigen-specific IgE [11]. In addition, diosgenin attenuated allergic inflammation and augmented regulatory immunity in the gut of mice with food allergy [12]. Although several studies have demonstrated the induction of Th1-like Treg cells under Th1 polarizing conditions, evidence pertaining to the induction of Th1-like Treg cells under Th2 polarizing conditions is scarce [8,9,14–17]. Since diosgenin enhanced the functionality of both Th1 and Treg cells, we hypothesized that diosgenin may enhance the induction of Th1-like Treg cells in the intestine of Th2-type allergic mice. To test this hypothesis, we characterized the transcription factors and cytokines of both Th1 and Treg cells in the intestine and Peyer's patches of allergic mice treated with diosgenin. The chemokine network associated with the recruitment of Th1-like Treg cells was also examined.

2. Materials and methods

2.1. Reagent, chemicals and antibodies

All reagents, including diosgenin (purity $\geq 99\%$) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise stated. Cell culture supplies and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Reagents used for immunohistochemical (IHC) staining were purchased from BioGenex Laboratories (San Ramon, CA, USA) and AbCam Inc. (Cambridge, MA, USA). The following purified antibodies were used for IHC staining: rat anti-mouse IFN- γ IgG₁, rat anti-mouse Foxp3 IgG_{2b}, rat anti-mouse IL-10 IgG_{2b} (BioLegend, San Diego, CA, USA), rat anti-mouse T-bet IgG, rat anti-mouse CXCR-3 IgG_{2a} (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-mouse CCR5 IgG (GeneTex, San Antonio, TX, USA). The anti-rat IgG secondary antibodies conjugated with alkaline phosphatase (AP) or horse radish peroxidase (HRP) were purchased from AbCam, Inc.

2.2. Animals, ethical aspects and in vivo procedures

Male BALB/c mice (5 weeks old) were obtained from the Animal Breeding Center of the National Taiwan University Hospital (Taipei, Taiwan) and housed in a temperature ($25 \pm 2^\circ\text{C}$), humidity ($50 \pm 20\%$), and light-controlled environment (12 h light/dark cycle). Food and water were supplied ad libitum, except on the days of allergen challenge (described in the following section).

A previously described murine model of food allergy was employed [11,12]. Mice were randomly divided into the following groups: naïve (NA), nonsensitized (NS), ovalbumin-sensitized and challenged (OVA), vehicle-treated and ovalbumin-sensitized and challenged (VH), and diosgenin-treated and ovalbumin-sensitized and challenged (DIO). Diosgenin was suspended in olive oil (vehicle) as a fine suspension. Diosgenin (100 and 200 mg/kg; D100 and D200; 0.1 mL per mouse) and VH (0.1 mL per mouse) were administered daily by gavage throughout the experiment. Except for the NA and NS groups, each mouse was sensitized with ovalbumin by intraperitoneal injection using 0.1 mL sensitization solution containing 50 μg ovalbumin and

1 mg aluminum potassium sulfate on day 3 and boosted on day 17. To induce allergic responses, the mice (except for NA) were repeatedly challenged with ovalbumin (50 mg dissolved in 0.3 mL saline per mouse) by gavage every other day from day 31 to day 43. Mice were deprived of food 3 h before diosgenin administration on the days of challenge and were challenged 1 h after diosgenin administration. Allergic diarrhea, which was identified as profuse liquid stool, was monitored visually for 3 h after OVA challenge. Before euthanization, the serum samples from mice were collected for OVA-specific IgE and total IgE detection by ELISA as previously described [11]. All mice were euthanized 3 h after the last challenge, and the duodenal tissues and Peyer's patches were isolated for further experiments (Fig. 1). The animal experiments were approved by the institutional Animal Care and Use Committee of the National Taiwan University (NTU-97-EL-71).

2.3. Cell preparation and flow cytometric analysis

Peyer's patches were excised from the proximal and distal parts of the small intestines of mice and kept in cold RPMI 1640 medium containing 10% FBS. Single-cell suspensions were prepared by gently teasing the tissues and passing the cells through a nylon mesh cell strainer (BD Pharmingen, San Jose, CA, USA). After washing, Peyer's patches cells (5×10^6 cells/mL) were seeded in 60-mm culture dishes (2 mL per dish) and stimulated for 8 h with phorbol-12-myristate-13-acetate (80 nM) plus ionomycin (1 μM) in the presence of GolgiStop (BD Biosciences). The cells were fixed and permeabilized using the Fixation/Permeabilization buffer (eBioscience, San Diego, CA, USA) following the supplier's instructions. The cells were then stained with FITC-conjugated anti-IL-10, PE-conjugated anti-Foxp3, PerCP/Cy5.5-conjugated anti-T-bet, PerCP/Cy5.5-conjugated anti-CXCR3 and PerCP/Cy5.5-conjugated anti-IFN- γ (Biolegend, San Diego, CA, USA), and analyzed using a flow cytometer (BD FACSCalibur, San Jose, CA, USA).

2.4. Immunohistochemical (IHC) double staining

The duodenal tissues (1.5 to 4.5 cm toward the anal side from the pylorus) were excised and fixed in 10% neutral buffered formalin. Tissue

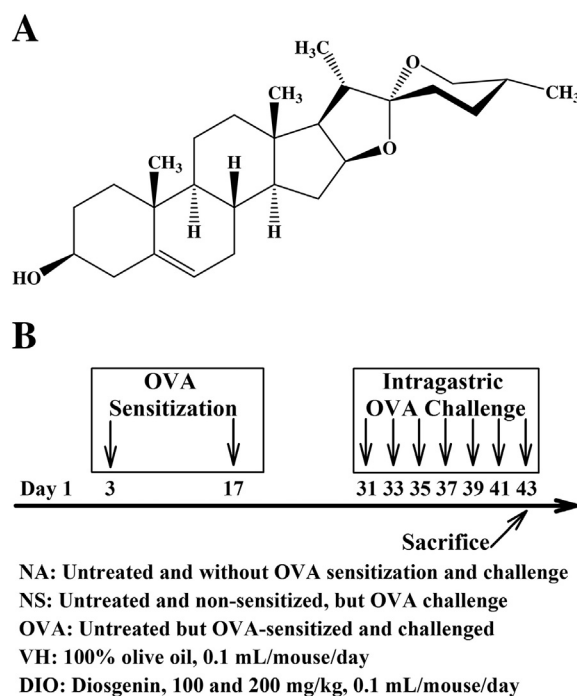


Fig. 1. (A) Chemical structure of diosgenin and (B) protocols of diosgenin administration and ovalbumin sensitization and challenge as described in the methods.

blocks were embedded in paraffin, cut into 4–5 μm thick sections on silane coated slides, and subjected to IHC double staining as described previously [12]. For dewaxing, slides were immersed in xylene for 5 min three times and then immersed sequentially in 100%, 95%, 90%, 80%, and 60% ethanol for 5 min each for hydration. The hydrated slides were immersed in Trilogy (Cell Marque Corporation, Rocklin, CA) at 121 °C for 15 min for antigen retrieval. The slides were then treated with 3% H_2O_2 for 15 min followed by blocking with normal horse serum for 1 h. After washing, the slides were incubated with primary antibodies at 4 °C in the dark overnight, treated with Super Enhancer reagent for 1 h, and then incubated with Poly-HRP reagent or HRP-conjugated secondary antibody for 1 h. For visualization, the slides were treated with the HRP substrate 3-amino-9-ethylcarbazole (AEC) for 2 min. AEC-treated slides were then incubated with another primary antibody at 4 °C in the dark overnight followed by incubation with AP-conjugated secondary antibody for 1 h. Finally, the slides were treated with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium, a substrate of AP) solution for 30 min for observation of a second staining without counter-staining. The slides were washed three times between each step after dewaxing. Except for the step of antigen retrieval and incubation with primary antibodies, each step was operated in the dark at room temperature. The number of IHC-single positive cells was quantified using the Image-Pro Plus 5.1 morphometric program (Media Cybernetics Inc., Rockville, MD, USA), and double positive signals were counted manually. Six measurements per tissue segment and 6–9 segments per group were analyzed at 200- or 400-fold magnification.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the duodenal tissues without the Peyer's patches was extracted using TRI Reagent (Sigma-Aldrich) following the supplier's instructions. The steady state mRNA expression of CXCR3, CCL3, CXCL10 and β -actin was measured by RT-PCR. All isolated RNA samples were confirmed to be free of DNA contamination as determined by the absence of products after PCR amplification in the absence of RT. For reverse-transcription, 2 μg of total RNA were reverse transcribed into cDNA by 40 units MMLV reverse transcriptase using oligo-dT as primers. The reverse transcription proceeded at 42 °C for 15 min and then at 95 °C for 5 min. A PCR master mixture consisting of PCR buffer, 4 mM MgCl_2 , a 6 pmol each of forward and reverse primers specific for the gene of interest, and 2.5 units of Taq DNA polymerase were added to each cDNA sample for PCR. The PCR primers used are: CXCR3, 5'-AACAGCACCTCTCCCTACGA-3', and 5'-AAGGCCCTGCATAGAAGTT-3'; CCL3, 5'-GCAGCGAGGAACCCCCAAA-3', and 5'-TTGAGG-AACGTGTCCTGAAGT-3'; CXCL10, 5'-GAATCCGGAATCTAAGACCA-3', and 5'-GTAGCACTGATGTGACC-3'; β -actin, 5'-AGGGAATCGTGCCTGACATCAAA-3' and 5'-ACTCATCGTACTCTGCTGCTGA-3' [18]. PCR products were electrophoresed in 1.5% agarose gels, and visualized by ethidium bromide staining. Quantification was performed by assessing the optical density for the DNA bands (interested cytokine mRNA) using the alpha imager 1200 digital imaging system (Alpha Innotech Corp, San Leandro, CA, USA). The results are expressed as the density ratio between the interested gene and the reference standard (β -actin).

2.6. Statistical analysis

Data are expressed as mean $\pm$ standard error (SE) for each treatment group in the individual experiments. Normality and homoscedasticity of data were tested by the Shapiro-Wilk test and the Bartlett's test, respectively. Analysis of variance was performed by one-way ANOVA. Dunnett's two-tailed *t*-test was employed to compare diosgenin-treated groups to the vehicle control group. *p* value <0.05 was defined as statistical significance.

3. Results

3.1. Diosgenin increases the number of Th1-like Treg cells in the duodenum of allergic mice

The anti-allergic effects of diosgenin observed in this study was similar to those demonstrated in our previous studies [11]. Elevated levels of serum IgE and the increased occurrence of allergic diarrhea were observed in mice sensitized and challenged with OVA, which were significantly attenuated by diosgenin treatment (Fig. S1). These results confirmed that the allergy model was successfully established, and diosgenin treatment attenuated food allergic responses.

To investigate the effect of diosgenin administration on the induction of Th1-like Treg cells, IHC staining was employed to detect the phenotypic and functional features of Th1 and Treg cells in the duodenum. A decreased number of Foxp3⁺ cells was observed in the OVA group (Table 1; OVA vs. NS), showing a down-regulated regulatory immunity in mice with food allergy. Consistent with our previous results [12], diosgenin treatment increased the number of Foxp3⁺ and Foxp3⁺ IL-10⁺ cells (Table 1; DIO vs. VH). The number of T-bet⁺ and IFN- γ ⁺ cells was also increased in diosgenin-treated groups (Table 1). Notably, the number of Treg cells co-expressing Th1 markers, including Foxp3⁺ IFN- γ ⁺ and Foxp3⁺ T-bet⁺, was also markedly increased by diosgenin treatment (Fig. 2, Fig. S2 and Table 1). Moreover, diosgenin increased the number of Th1 cells co-expressing the regulatory cytokine IL-10, including IL-10⁺ IFN- γ ⁺ and IL-10⁺ T-bet⁺ cells (Table 1 and Fig. S2). The expression of TGF- β , another major regulatory cytokine, was comparable between groups (Table 1).

3.2. Diosgenin increases the number of Th1-like Treg cells in the Peyer's patches

Peyer's patches are the intestinal lymphoid tissues where the induction of antigen-specific immune responses and the generation of IL-10-secreting Treg cells occur [19,20]. Hence, we investigated whether diosgenin induced Th1-like Treg cells in the Peyer's patches. The population of Foxp3⁺ cells in the Peyer's patches was significantly increased in diosgenin-treated groups, as compared to the VH group (Fig. 3). Notably, a significantly increased populations of Foxp3⁺ cells co-expressing both IL-10 and IFN- γ as well as Foxp3⁺ cells co-expressing both

Table 1

Immunohistochemical staining of Foxp3⁺, IL-10⁺, T-bet⁺, IFN- γ ⁺, CXCR3⁺ and CCR5⁺ cells in the duodenum.

	Number of positive cells (cells per mm ²)					
	NA	NS	OVA	VH	D100	D200
Foxp3 ⁺	38 $\pm$ 2	36 $\pm$ 4	28 $\pm$ 3 [#]	29 $\pm$ 1	42 $\pm$ 4 [*]	38 $\pm$ 4 [*]
TGF- β	24 $\pm$ 3	24 $\pm$ 3	20 $\pm$ 3	21 $\pm$ 2	25 $\pm$ 2	22 $\pm$ 4
IL-10 ⁺	15 $\pm$ 1	16 $\pm$ 2	32 $\pm$ 2 [#]	30 $\pm$ 3	42 $\pm$ 4 [*]	42 $\pm$ 3 [*]
IFN- γ ⁺	17 $\pm$ 3	18 $\pm$ 2	24 $\pm$ 3 [#]	24 $\pm$ 2	35 $\pm$ 1 [*]	34 $\pm$ 4 [*]
T-bet ⁺	12 $\pm$ 2	12 $\pm$ 3	14 $\pm$ 2	13 $\pm$ 1	21 $\pm$ 2 [*]	20 $\pm$ 2 [*]
CCR5 ⁺	12 $\pm$ 5	15 $\pm$ 5	14 $\pm$ 2	18 $\pm$ 4	25 $\pm$ 4 [*]	28 $\pm$ 2 [*]
CXCR3 ⁺	14 $\pm$ 2	13 $\pm$ 2	19 $\pm$ 2 [#]	21 $\pm$ 2	26 $\pm$ 2 [*]	28 $\pm$ 2 [*]
Foxp3 ⁺ IL-10 ⁺	10 $\pm$ 1	14 $\pm$ 2	7 $\pm$ 1	8 $\pm$ 2	23 $\pm$ 5 [*]	20 $\pm$ 4 [*]
Foxp3 ⁺ IFN- γ ⁺	11 $\pm$ 1	14 $\pm$ 1	11 $\pm$ 1	12 $\pm$ 2	20 $\pm$ 1 [*]	22 $\pm$ 2 [*]
Foxp3 ⁺ T-bet ⁺	3 $\pm$ 1	2 $\pm$ 0	2 $\pm$ 1	3 $\pm$ 0	6 $\pm$ 1 [*]	6 $\pm$ 1 [*]
Foxp3 ⁺ CCR5 ⁺	5 $\pm$ 3	6 $\pm$ 3	8 $\pm$ 2	10 $\pm$ 4	16 $\pm$ 3 [*]	15 $\pm$ 2 [*]
Foxp3 ⁺ CXCR3 ⁺	7 $\pm$ 1	10 $\pm$ 1	8 $\pm$ 1	10 $\pm$ 1	18 $\pm$ 1 [*]	19 $\pm$ 2 [*]
IL-10 ⁺ IFN- γ ⁺	2 $\pm$ 0	2 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1	10 $\pm$ 1 [*]	8 $\pm$ 2 [*]
IL-10 ⁺ CXCR3 ⁺	2 $\pm$ 0	3 $\pm$ 1	3 $\pm$ 0	3 $\pm$ 1	9 $\pm$ 2 [*]	8 $\pm$ 2 [*]
IL-10 ⁺ T-bet ⁺	2 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	2 $\pm$ 1	4 $\pm$ 1 [*]	4 $\pm$ 1 [*]

The number of single positive cells was quantified using Image-Pro Plus 5.1 morphometric software, and double positive cells were counted manually. Data are expressed as mean $\pm$ SEM (n = 3). [#]*p* < 0.05 compared to the NS group. ^{*}*p* < 0.05 compared to the VH group. Results are a representative of 3 independent experiments.

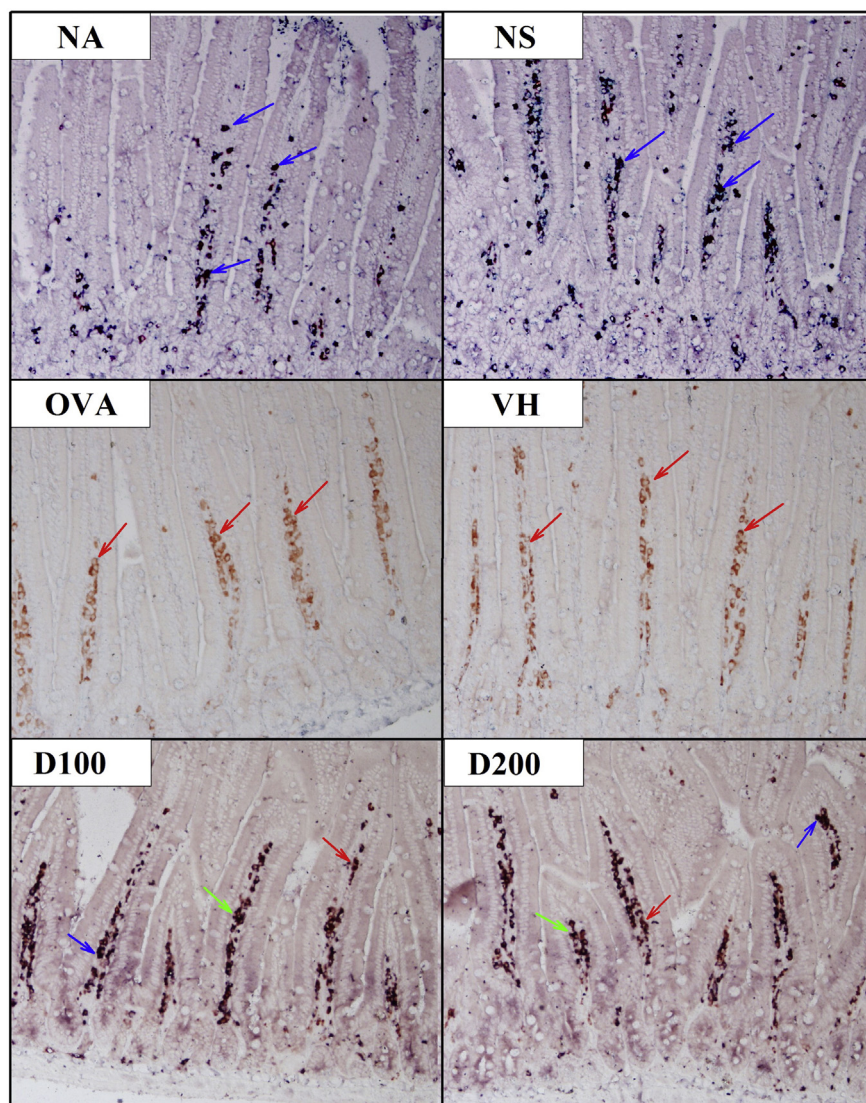


Fig. 2. Diosgenin increased the number of intestinal Foxp3⁺ IFN- γ ⁺ cells. The duodenal tissues from each group were processed and stained as described in the methods. Representative duodenal sections stained with Foxp3 and IFN- γ are shown (original magnification, $\times 200$). Blue, red and green arrows indicate Foxp3⁺, IFN- γ ⁺, and Foxp3⁺ IFN- γ ⁺ cells, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

IL-10 and T-bet were observed in diosgenin-treated groups (Fig. 3A and B).

3.3. Diosgenin increases the expression of Th1-associated chemokine receptors on Treg cells and Th1-associated chemokines in the duodenum

To investigate whether diosgenin affects the recruitment of Th1-like Treg cells in the duodenum, two Th1-associated chemokine/chemokine receptor pairs were determined. Firstly, the number of Foxp3⁺ CXCR3⁺ and Foxp3⁺ CCR5⁺ cells was determined by IHC double staining to decipher if the expression of Th1-associated chemokine receptors on Treg cells was altered. Diosgenin significantly increased the number of both Foxp3⁺ CXCR3⁺ and Foxp3⁺ CCR5⁺ cells in the duodenum (Table 1). The enhanced expression of CXCR3 in diosgenin-treated groups was further confirmed at the mRNA level (Fig. 4A). Concordantly, the expression of CXCL10 and CCL3, the corresponding chemokines for CXCR3 and CCR5, in the duodenum was also up-regulated by diosgenin (Fig. 4B and C). In line with the enhanced expression of CXCR3 in the intestine, further characterization of the Foxp3⁺ cells in the Peyer's patches revealed that a significantly increased populations of Foxp3⁺ cells co-expressing both IL-10 and CXCR3 was observed in diosgenin-treated groups (Fig. 5). These results showed that diosgenin

enhanced the migration of functional Treg cells to the sites challenged with antigen via the Th1-type chemokine network.

4. Discussion

Diosgenin has been demonstrated to possess anti-allergic and immunomodulatory activities. However, the effects of diosgenin on the induction of Th1-like Treg cells and the role of Th1-like Treg cells in diosgenin-mediated anti-allergic effects have not been addressed. In the present study, we demonstrated that oral intake of diosgenin enhanced the induction of Th1-like Treg cells in the gut of mice with food allergy, a Th2-dominant allergic condition. This notion is substantiated by several lines of evidence. Firstly, diosgenin increased the number of Foxp3⁺ Treg cells co-expressing Th1 markers, including CCR5, CXCR3, IFN- γ and T-bet, in the intestine. Secondly, diosgenin increased the population of Foxp3⁺ IFN- γ ⁺ and Foxp3⁺ T-bet⁺ cells that expressed the regulatory cytokine IL-10 in the Peyer's patches. Lastly, an increase in the number of CXCR3⁺ cells co-expressing Foxp3 and IL-10 was detected in the Peyer's patches of diosgenin-treated mice. These data clearly demonstrated an enhanced induction of a population of T cells exhibiting both Treg and Th1 features by diosgenin, providing

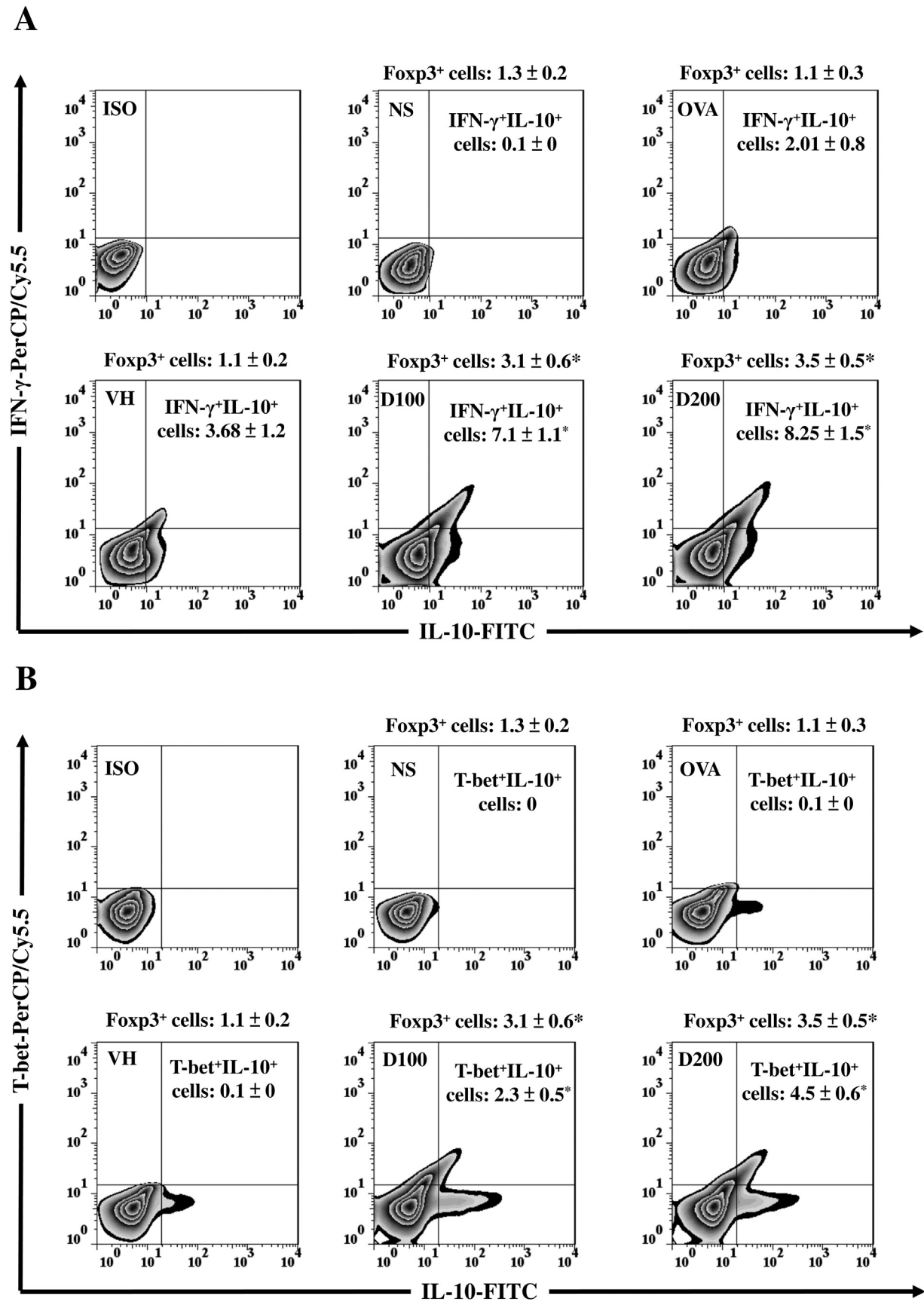


Fig. 3. Diosgenin induced IFN- γ ⁺ and T-bet⁺ Treg cells in Peyer's patches. Peyer's patch cells were fixed and permeabilized for three-color flow cytometric detection of intracellular cytokines and transcription factors as described in the methods. The cells were stained with FITC-conjugated anti-IL-10, PE-conjugated anti-Foxp3 and either PerCP/Cy5.5 anti-IFN- γ or PerCP/Cy5.5 anti-T-bet. The Foxp3-gated cells were analyzed for expression of (A) IL-10 and IFN- γ , or (B) IL-10 and T-bet. Numbers in quadrants represent the percentage of triple positive cells. **p* < 0.05 compared to the VH group. Results are a representative of 3 independent experiments.

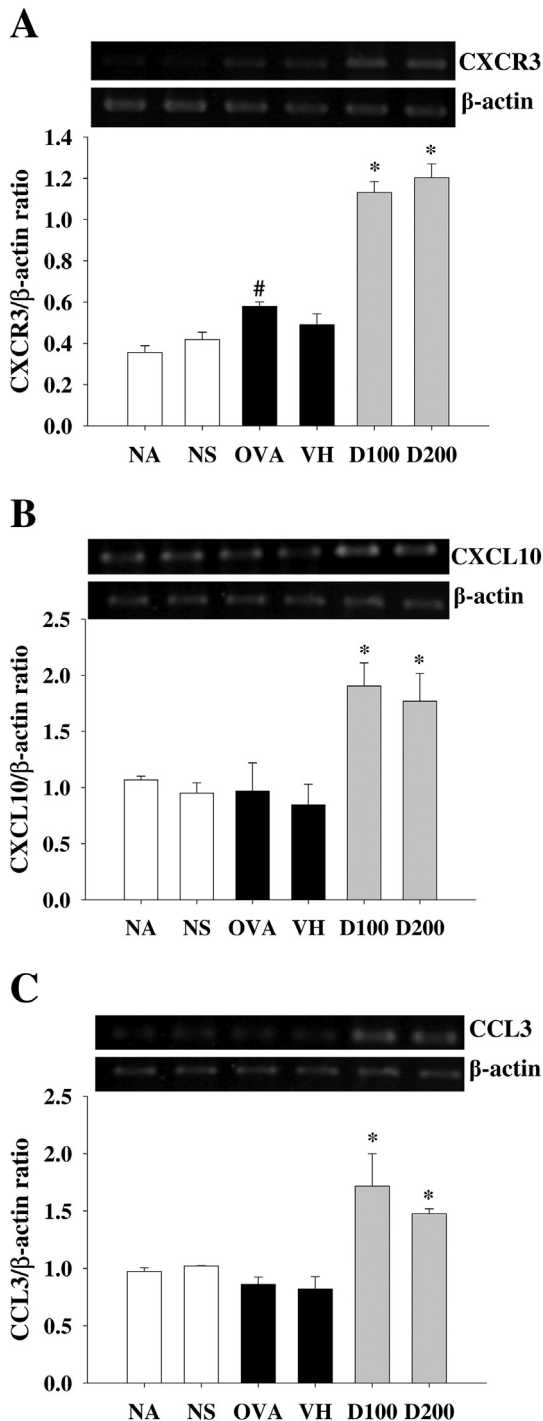


Fig. 4. Diosgenin enhanced the expression of Th1-associated chemokines and chemokine receptor CXCR3 in the duodenum. The level of (A) CXCR3, (B) CCL3 and (C) CXCL10 mRNA expression in the duodenum was determined by RT-PCR. The results are quantified as the density ratio between the gene of interest and the reference standard β -actin. Representative photos of RT-PCR products are shown in the upper panel. Data are expressed as means $\pm$ SEM ($n = 4$). [#] $p < 0.05$ compared to the NS group. ^{*} $p < 0.05$ compared to the VH group. Results are a representative of 3 independent experiments.

critical insights to the underlying immunological mechanisms of diosgenin-mediated anti-allergic effects.

The induction of Th1-like Treg cells has been shown to be a crucial mechanism effective for ameliorating certain immune disorders. For example, Foxp3⁺T-bet⁺ Treg cells expressing both IL-10 and IFN- γ have been identified in antigen- and heat-killed *Listeria monocytogenes*-primed mice, which contributes to the suppression of airway

hyperreactivity [16]. During parasitic infection, parasite-driven Th1-polarized environment promoted the development of a corresponding Th1-like Treg cell population to limit Th1-type inflammation [14]. In rat studies, Th1-like Treg cells induced from naïve natural Treg cells cultured with alloantigens were capable of delaying allograft rejection [17]. In non-obese diabetic mice, Th1-Like ICOS⁺Foxp3⁺ Treg cells expressing CXCR3 could potentially dampen inflammation and control autoimmune diabetes [15]. Collectively, these reports indicate that Th1-polarizing conditions, such as pathogen infection, graft rejection or autoimmunity, may enhance the induction of Th1-like Treg cells to counteract overactive Th1 immune responses. The difference between previous reports and the present study is that diosgenin suppressed food allergic responses in association with the induction of Th1-like Treg cells, rather than a severe pro-inflammatory status caused by pathogen infection or Th1-mediated autoimmune diseases. Our results showed that immunoactive phytochemicals, such as diosgenin, could enhance the induction of Th1-like Treg cells in allergic mice. It is suggested that diosgenin-mediated induction of Th1-like Treg cells in the gut may be a potential strategy to counteract the aberrant immunobalance of Th2-associated disorders, such as food allergy. To date, the exact role of Th1-like Treg cells in the pathophysiology of food allergy and the gut immunobalance has not been completely elucidated. The interaction between Th1-like Treg cells and the intestinal immunocompetent cells mediating allergic reactions is an intriguing issue that warrants further investigation.

The expression of chemokines by intestinal epithelial cells plays a crucial role for maintaining the enteric immunobalance [21,22]. Differential expression of chemokine receptors on Treg cells allows their trafficking to different target sites [23]. For example, CXCR3 plays a key role to guide effector Treg cells trafficking to Th1 chemokine-rich target sites to ameliorate Th1 cell-mediated inflammation, highlighting a crucial role for CXCR3 in the localization and functionality of not only the Th1 but also the Treg cells [15,24,25]. In the present study, the number of CXCR3⁺ and CCR5⁺ Treg cells, and the expression of their corresponding chemokines CXCL10 and CCL3 in the duodenum were significantly increased in diosgenin-treated mice. Concordantly, diosgenin increased the number of CXCR3⁺Foxp3⁺IL-10 cells in the Peyer's patches. Taken together, these findings suggest that an enhanced cell trafficking via the CXCR3-mediated signaling may contribute to the induction of intestinal Th1-like Treg cells in the gut of diosgenin-treated mice. To date, the role of CCR5 for the development of Th1-like Treg cells remains unclear, which warrants further investigations.

Although some herbal and natural supplements are recognized as causing hepatotoxicity, there is a clear lack of data studying the safety of diosgenin per se [26]. Despite the fact that the doses of diosgenin used in the present study are far lower than the oral LD₅₀ in mice (>8 g/kg) [27], further studies are warranted to clarify the potential adverse effects of diosgenin at the employed doses.

In conclusion, we demonstrated that diosgenin treatment enhanced the induction of Th1-like Treg cells in the gut of mice with food allergy. The present results provide the first evidence to show the in vivo induction of Th1-like Treg cells under a Th2-type allergic condition, and suggest that an enhanced induction of Th1-like Treg cells by diosgenin may be employed as a novel strategy to prevent or ameliorate intestinal allergy.

Conflict of interest

The authors declare no conflicts of interest relevant to this work.

Author contributions

Chung-Hsiung Huang and Chia-Chi Wang performed the experiments and acquired the data. Tong-Rong Jan conceived and designed the study. Masatoshi Hori and Yu-Chin Lin contributed to data analysis and manuscript discussion.

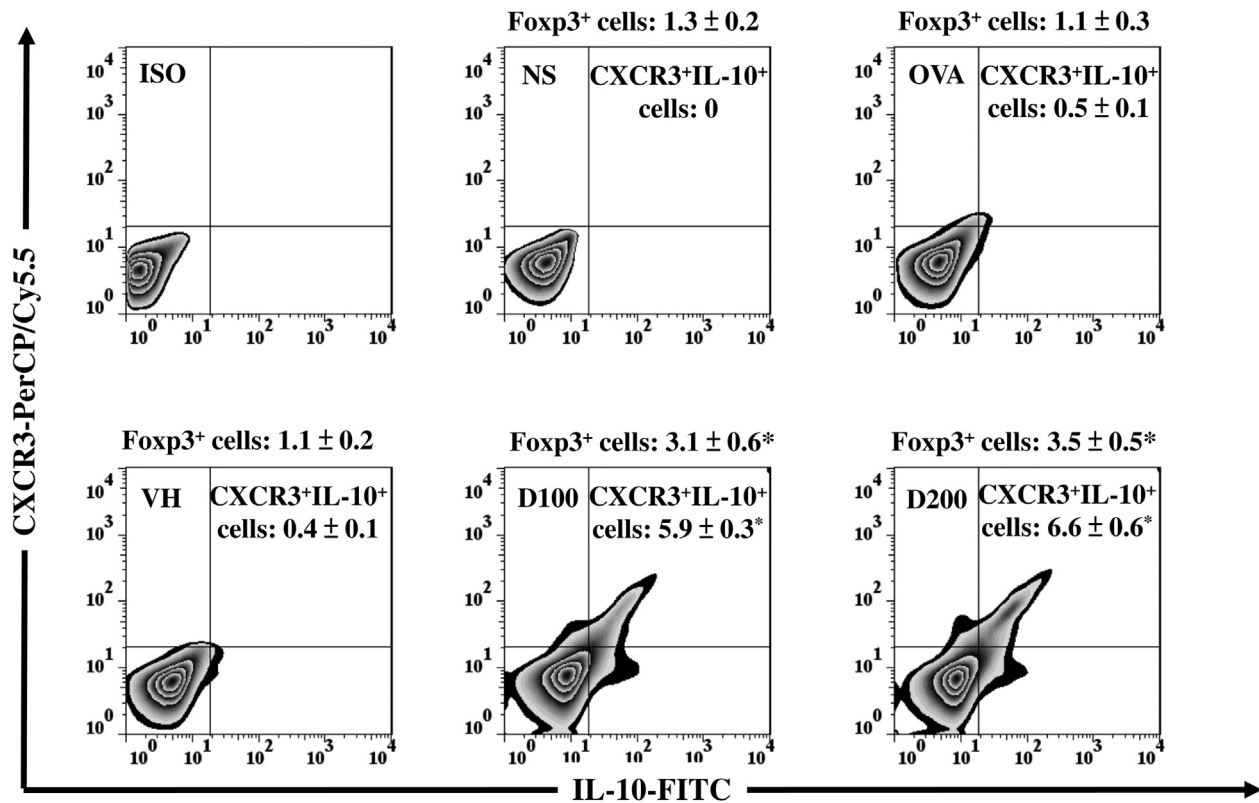


Fig. 5. Diosgenin induced CXCR3⁺ Treg cells in Peyer's patches. Peyer's patch cells were fixed and permeabilized for three-color flow cytometric detection of intracellular cytokines and transcription factors as described in the methods. The cells were stained with FITC-conjugated anti-IL-10, PE-conjugated anti-Foxp3 and PerCP/Cy5.5 anti-CXCR3. The Foxp3-gated cells were analyzed for expression of IL-10 and CXCR3. Numbers in quadrants represent the percentage of Foxp3⁺ cells co-expressing IL-10 and CXCR3. **p* < 0.05 compared to the VH group. Results are a representative of 3 independent experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2016.11.021>.

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Supplementary data

Oral administration with diosgenin enhances the induction of intestinal T helper 1-like regulatory T cells in a murine model of food allergy

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Figure legends

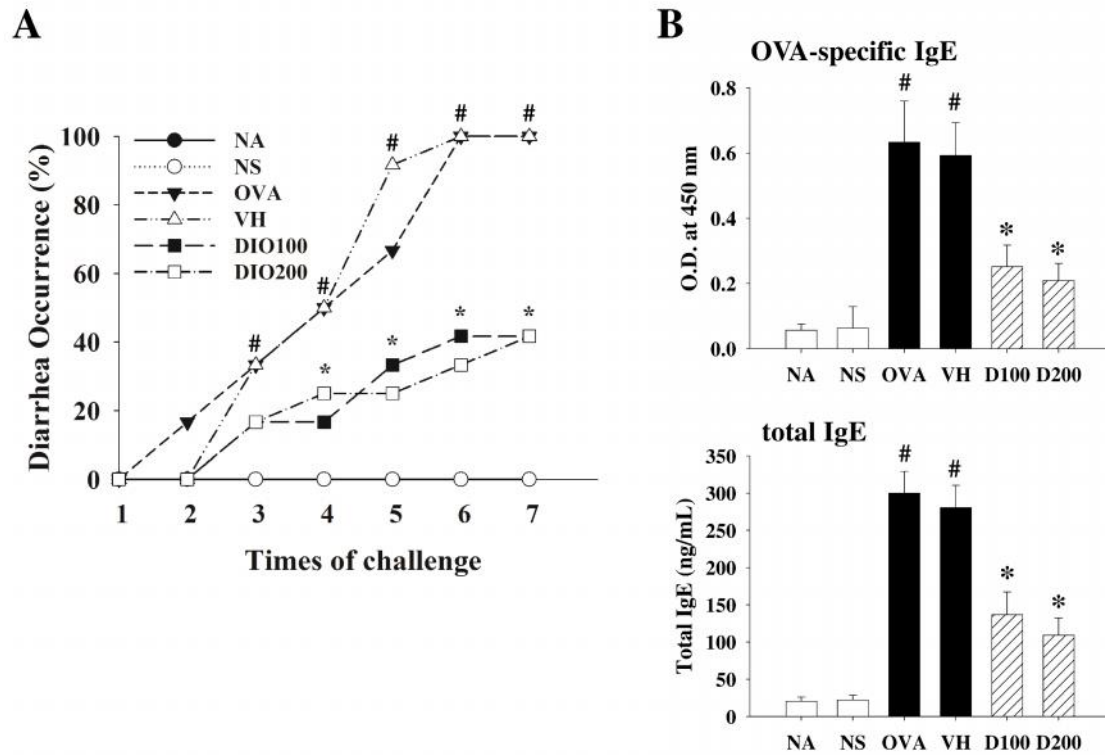


Fig. S1. Diosgenin treatment attenuated allergic responses. (A) After each OVA challenge, diarrhea was monitored visually for 3 h. Mice with profuse liquid stool were identified as diarrhea-positive ones. ●, NA (n=9); ○, NS (n=9); ▼, OVA (n=12); △, VH (n=12); ■, D100 (n=12); □, D200 (n=12). (B) Serum samples were collected before sacrifice to detect the levels of total IgE and OVA-specific IgE production by ELISA. Data are pooled from 3 independent experiments and expressed as mean ± S.E.M. of 9–12 mice per group. #, $p < 0.05$ compared to the NS group; *, $p < 0.05$ compared to the VH group.

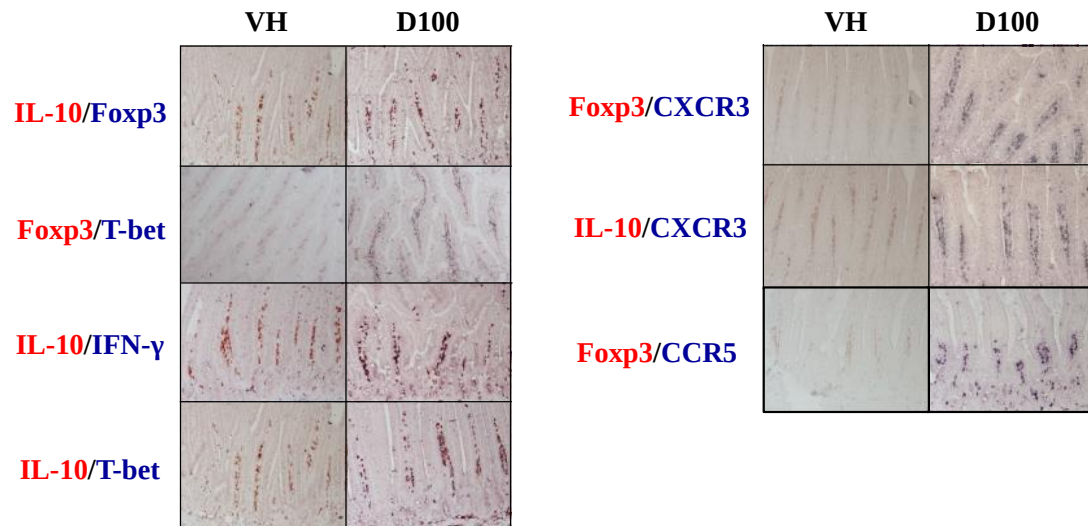


Fig. S2. Representative duodenal sections of IHC double staining are shown (original magnification, $\times 200$). The red and dark blue signals are indicated by the labels shown.