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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.

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-overeactive 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 ≥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 IgG_1, 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 ± 2 °C), humidity (50 ± 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

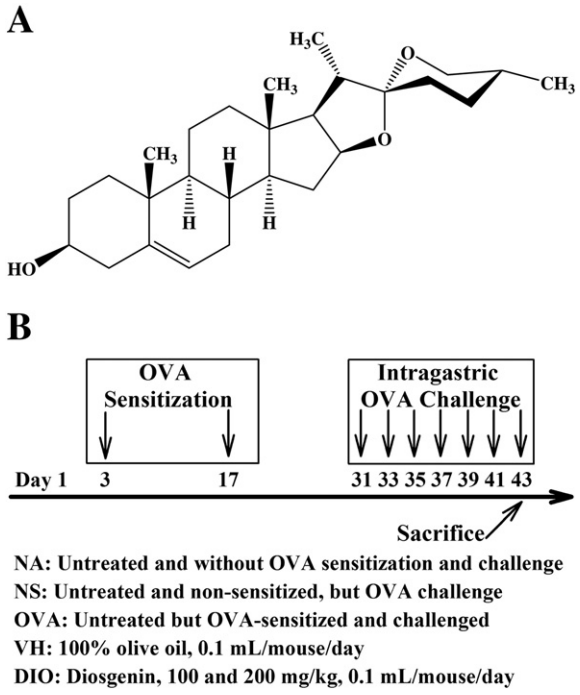

Fig. 1. (A) Chemical structure of diosgenin and (B) protocols of diosgenin administration and ovalbumin sensitization and challenge as described in the methods.