



Research article

Enhanced Rg3 negatively regulates Th1 cell responses

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ABSTRACT

Background: Korean Red Ginseng (KRG; *Panax ginseng* Meyer) is a widely used medicinal herb known to exert various immune modulatory functions. KRG and one of its purified components, ginsenoside Rg3, are known to possess anti-inflammatory activities. How they impact helper T cell-mediated responses is not fully explored. In this study, we attempted to evaluate the effect of KRG extract (KRGE) and ginsenoside Rg3 on Th1 cell responses.

Methods: Using well-characterized T cell *in vitro* differentiation systems, we examined the effects of KRGE or enhanced Rg3 on the Th1-inducing cytokine production from dendritic cells (DC) and the naïve CD4⁺ T cells differentiation to Th1 cells. Furthermore, we examined the change of Th1 cell population in the intestine after treatment of enhanced Rg3. The influence of KRGE or enhanced Rg3 on Th1 cell differentiation was evaluated by fluorescence-activated cell sorting, enzyme-linked immunosorbent assay, and quantitative real-time polymerase chain reaction.

Results: KRGE significantly inhibited the production level of IL-12 from DCs and subsequent Th1 cell differentiation. Similarly, enhanced Rg3 significantly suppressed the expression of interferon gamma (IFN γ) and T-bet in T cells under Th1-skewing condition. Consistent with these effects *in vitro*, oral administration of enhanced Rg3 suppressed the frequency of Th1 cells in the Peyer's patch and lamina propria cells *in vivo*.

Conclusion: Enhanced Rg3 negatively regulates the differentiation of Th1 cell *in vitro* and Th1 cell responses in the gut *in vivo*, providing fundamental basis for the use of this agent to treat Th1-related diseases.

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1. Introduction

Korean Red Ginseng (KRG) is heat-processed White ginseng (the root of *Panax ginseng* Meyer); it has better pharmacological activities and has enhanced preservation efficacy and safety [1,2]. Accumulating evidence clearly demonstrates the beneficial effects of KRG extract (KRGE) on enhancing immune functions [3,4] as well as ameliorating diverse diseases including diabetes [5,6], colitis [7,8], cancer [7,9], atherosclerosis [10,11], neurodegenerative disease [12,13], and stress [14].

Various pharmacological components are examined in ginseng extract such as acidic polysaccharides, ginsenosides, polyacetylenes, and polyphenolic compounds [15]. Among them, ginsenosides have been thought to be important ingredients, which provide ginseng's pharmacological and biological activities [12,16,17]. Multiple types of ginsenosides are present in ginseng

extracts (e.g., Rbs, Rcs, Rd, Re, Rfs, Rgs); among them, ginsenoside Rg3 (40.1%), Rg5 (18.6%), and Rh2, Rk1 (5.73%), and Rs4 are uniquely found in Red ginseng [18,19]. In particular, Rg3 has been reported to prevent or ameliorate diseases, such as chronic fatigue [20], diabetes [21], and tumor [22].

On the other hand, dendritic cells (DC) are professional antigen-presenting cells (APCs) that connect innate and adaptive immune responses [23,24]. Once DCs uptake antigens, DCs produce pro-inflammatory cytokines, increase the co-stimulatory molecules, and subsequently present antigens to T cells [23–25]. Of note, ginseng extract or ginsenosides have been shown to modulate the maturation and function of DCs. For instance, ginseng saponins or ginseng metabolites enhanced DC maturation markers, such as CD80, CD83, CD86, and MHCII [26,27]. In addition, ginseng activated DCs to produce IL-1 and TNF α , and ginseng-primed DCs improved the CD4⁺ T cell proliferations and the interferon gamma

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(IFN γ) production [27,28]. However, several reports have shown opposite effects of ginseng on DCs including the diminished production of IL-12 and TNF- α and the inhibition of CD40 and CD86 expression [29,30].

The preventive and therapeutic effects of whole ginseng extract or ginsenosides on various immune disorders have been reported in several studies [6,8,13,31]; however, the effect of ginsenosides on the development of each subset of T cells remains incompletely understood. In this present study, we investigated the influence of ginsenoside Rg3 on Th1 cell responses *in vitro* and *in vivo*. Our findings demonstrate that Rg3 attenuates the differentiation of IFN γ ⁺ CD4⁺ T cell *in vitro* and *in vivo*.

2. Materials and methods

2.1. Ethics statement

All vertebrate animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University (protocols SNU-170120-1) and were conducted in accordance with the guidelines of Seoul National University for the care and use of laboratory animals. All surgeries were performed under isoflurane anesthesia (Piramal Critical Care, Inc., PA, USA).

2.2. KRGE and enhanced Rg3

KRGE and enhanced Rg3 were provided by Korea Ginseng Corporation (Seoul, South Korea). Enhanced Rg3 is one of the KRGE that has been enriched in Rg3 content. In KRGE and enhanced Rg3, totally 1.94% and 5.425% of ginsenosides are contained, respectively. In case of KRGE, ginsenosides Rg1 (0.071%), Re (0.093%), Rf (0.121%), Rh1 (0.078%), Rg2(s) (0.192%), Rg2(r) (0.129%), Rb1 (0.462%), Rc (0.241%), Rb2 (0.183%), Rd (0.089%), Rg3(s) (0.214%), and Rg3(r) (0.091%) are included. In addition, enhanced Rg3 consists of nine ginsenosides: Rf (0.105%), Rh1 (0.294%), Rg2(s) (0.294%), Rb1 (0.290%), Rc (0.097%), Rb2 (0.197%), Rd (0.137%), Rg3(s) (3.373%), and Rg3(r) (0.638%). Enhanced Rg3 also has 4.77% of arginyl-fructosyl-glucose and 4.125% of acidic polysaccharides. The phytochemical study data was provided by The Korean Society of Ginseng. The KRGE and enhanced Rg3 were dissolved in sterilized water.

2.3. Cell cytotoxicity

To determine the proper doses of KRGE or enhanced Rg3, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reduction assay was performed. Briefly, increasing doses of KRGE (0 μ g/mL, 31.25 μ g/mL, 62.5 μ g/mL, 125 μ g/mL, 250 μ g/mL, and 500 μ g/mL) and enhanced Rg3 (0 μ M, 25 μ M, 50 μ M, 100 μ M, and 200 μ M) were treated with bone marrow-derived DCs for 24 h; the cells were then incubated with MTT solution (final conc. 500 μ g/mL) (Life Technologies, Carlsbad, CA, USA) for 4 h at 37°C. After incubation, MTT solution was removed and dimethyl sulfoxide was added. The absorbance was measured at 540 nm by a microplate reader (Bio-Rad, Hercules, CA, USA). Absorbances were normalized to untreated control to calculate cell viability.

2.4. Mice and *in vivo* experimental design

Ten C57BL/6 female mice aged 5 wk were purchased from Orient Bio (Seongnam-si, Gyeonggi, South Korea). These mice were divided into two groups, a control group and an enhanced Rg3-treated group. Mice were orally administered with distilled water (control) or enhanced Rg3 (0.03 gram/kg) every other day. After 4

wk, gut-draining mesenteric lymph nodes (MLNs), Peyer's patches (PPs), and large intestines were isolated for analysis of T cell subsets.

2.5. Generation of bone marrow-derived DCs

Bone marrow cells (3×10^6 cells) obtained from wild type mice were cultured in 3 mL of Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 10% fetal bovine serum (FBS), 55 μ M 2-mercaptoethanol, 2 mM L-glutamine, penicillin/streptomycin (all these were obtained from Gibco, Grand Island, NY, USA), and recombinant mouse Granulocyte-macrophage colony-stimulating factor (10 ng/mL; Peprotech, Seoul, South Korea). On Day 1, floating cells were discarded and fresh medium were added. Half of the medium was discarded and fresh medium was added every two days. On Day 7, cells were used as DCs.

2.6. Naïve T cell differentiation *in vitro*

Naïve CD4⁺ T cells (CD44^{high}CD62L^{low}CD25⁻) in spleen and lymph nodes of naïve mouse were isolated using a fluorescence-activated cell sorting (FACS) machine, FACSAria III (BD BioScience, San Jose, CA, USA). CD11c⁺ bone marrow-derived DCs were purified with CD11c microbeads. For Th1 cell differentiation, 1×10^4 CD11c⁺ DCs were co-cultured with 1×10^5 FACS-sorted CD4⁺ T cells in the presence of anti-CD3 ϵ (0.3 μ g/mL) antibody (145-2C11, BioXcell, West Lebanon, NH, USA), and lipopolysaccharide (LPS) (80 ng/mL) (Sigma, Seoul, South Korea) for 96 h. For APC-free Th1 cell differentiation, anti-CD3 ϵ (1 μ g/mL) and anti-CD28 (2 μ g/mL) (37.51, BioXcell) were pre-coated in a 96-well plate overnight at 4°C. After washing the plate with cold phosphate-buffered saline (PBS) three times, 1×10^5 naïve CD4⁺ T cells were stimulated with IL-2 (2 ng/mL) and IL-12 (10 ng/mL) (Peprotech) for 96 h. KRGE (250 μ g/mL) or various concentration of enhanced Rg3 (25 μ M, 50 μ M, and 100 μ M) were treated at the beginning of naïve CD4⁺ T cells.

2.7. Enzyme-linked immunosorbent assay

IL-1 β , 6, 12p70, 12p40, and TNF α in the culture supernatants of naïve T cell differentiation were quantified by enzyme-linked immunosorbent assay, according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

2.8. Real-time polymerase chain reaction

Total RNA from cells was isolated by TRIzol reagent (Ambion, CA, USA), and cDNA was synthesized with a cDNA Synthesis kit (Thermo Fisher Scientific Inc., New York, NY, USA). Relative gene expression levels were evaluated using SYBR Green (Bio-Rad) on ABI 7500 Fast Real-Time Polymerase Chain Reaction Systems (Applied Biosystems, Singapore). Target genes were normalized to the β -actin level in each sample. Primer sets for genes were synthesized at Cosmogentech (Seoul, South Korea): *Ifn* (sense, 5'-GATGCATTCATGAG-TATTGCCAAGT-3', antisense, 5'-GTGGACCACTCGGATGAGCTC-3'), *Eomes* (sense, 5'-TGAATGAACCTTCCAAGACTCAGA-3', antisense, 5'-GGCTTGAGGCAAAGTGTGACA-3'), *T-bet* (sense, 5'-CAACAACCCCTT TGCCAAAG-3', antisense, 5'-TCCCCAAGCAGTTGACAGT-3'), *Gata3* (sense, 5'-AGAACC GGCCCTTATGAA-3', antisense, 5'-AGTTCGCG-CAGGATGTCC-3'), *Rorc* (sense, 5'-CCGCTGAGAGGGCTTCAC-3', antisense, 5'-TG CAGGATAGCCACATTACA-3'), *Gzmb* (sense, 5'-GCCACAACATCAAAGAACAG-3', antisense, 5'-AACCAGCCACATAGC ACACAT-3'), β -actin (sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3', antisense, 5'-TAAACGCAGCTCAGTAACAGTCCG-3').

2.9. Flow cytometry

Cells were incubated for 3–4 h with 100 ng/mL of PMA, 1 μ M of ionomycin (all from Sigma), Brefeldin A and Monensin (all from eBioscience). After washing cells with cold PBS containing 1.5% FBS, the cells were stained with APC-Cy7-conjugated anti-CD4 mAb (eBioscience) for surface staining. Cells were then washed and stained with PerCp-Cy5.5-conjugated anti-IFN γ mAb, APC-conjugated anti-IL-17 mAb (all from BioLegend, San Diego, CA, USA), and Phycoerythrin (PE)-conjugated anti-T-bet mAb (eBioscience) after incubation with fixation/permeabilization buffer (eBioscience) for 30 min at 4°C (all from BioLegend). The cells were analyzed by flow cytometer, FACSVerse flow cytometer (BD Bioscience). Data were analyzed with FlowJo (TreeStar, Ashland, OR, USA).

2.10. Preparation of lamina propria cells

Large intestines were cut into 1 cm slices, and epithelium was removed by stirring in RPMI-1640 containing 1mM EDTA (Gibco) for 30 min and 2% FBS at 37°C (twice). After washing the gut pieces with pre-warmed PBS at least five times, they were cut into 1–2 mm and stirred into RPMI-1640 containing 2% FBS, 10 U/mL collagenase IV (Gibco), and 5 U/mL DNase I (Bio Basic Inc., Amherst NY, USA) for 30 min at 37°C (twice). After incubation, the suspension was filtered through a 100 μ m-pore nylon mesh (Small Parts Inc., FL, USA). The lymphocytes were purified by a 44%/70% Percoll (Pharmacia, Uppsala, Sweden) gradient.

2.11. Statistical analysis

All experiments were performed two to four times. Statistical analysis was conducted with mean \pm standard error of the mean by

unpaired two-tailed Student *t* test with Prism Graphpad 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. KRGE inhibits the pro-inflammatory cytokines produced by DCs

Previous studies have demonstrated that ginseng modulates immune responses and prevents immune disorders. The inhibitory efficacy of Red ginseng extracts on inflammation has been suggested to be better than that of White ginseng extracts [12,17,32]. However, the function of ginseng or its components to the differentiation of helper T cell subsets has been incompletely understood. As a first step to explore the immune modulatory functions of KRGE on helper T cell responses, we determined the effects of KRGE on the production of cytokines from DCs since they play an essential role in determining the type of effector T cells by acting as “signal 3” during the T cells differentiation [23–25]. In order to determine the proper dose of KRGE *in vitro* experiment, we conducted cell viability assay by MTT. The MTT reduction assay showed no reduction in cell viability under 250 μ g/mL (Fig. S1A). On the basis of this result, we used KRGE at the concentration of 250 μ g/mL. To investigate the effect of KRGE on the production of LPS-induced inflammatory cytokines, we stimulated bone marrow-derived DCs by LPS in the presence or absence of KRGE and measured the amounts of pro-inflammatory cytokines from DCs including IL-1 β , IL-6, IL-12, IL-23, and TNF α [24,33]. As shown in Fig. 1, KRGE itself had little role in inducing the production of pro-inflammatory cytokines from DCs. On the other hand, addition of KRGE slightly but significantly inhibited the production of IL-6, IL-10, IL-12p40, and TNF α induced by LPS (Fig. 1B–F). By contrast, the production of LPS-induced IL-1 β from DCs was slightly increased by KRGE (Fig. 1A). Since fermented White ginseng extracts (similar to Red ginseng extracts) has been shown to inhibit the production of

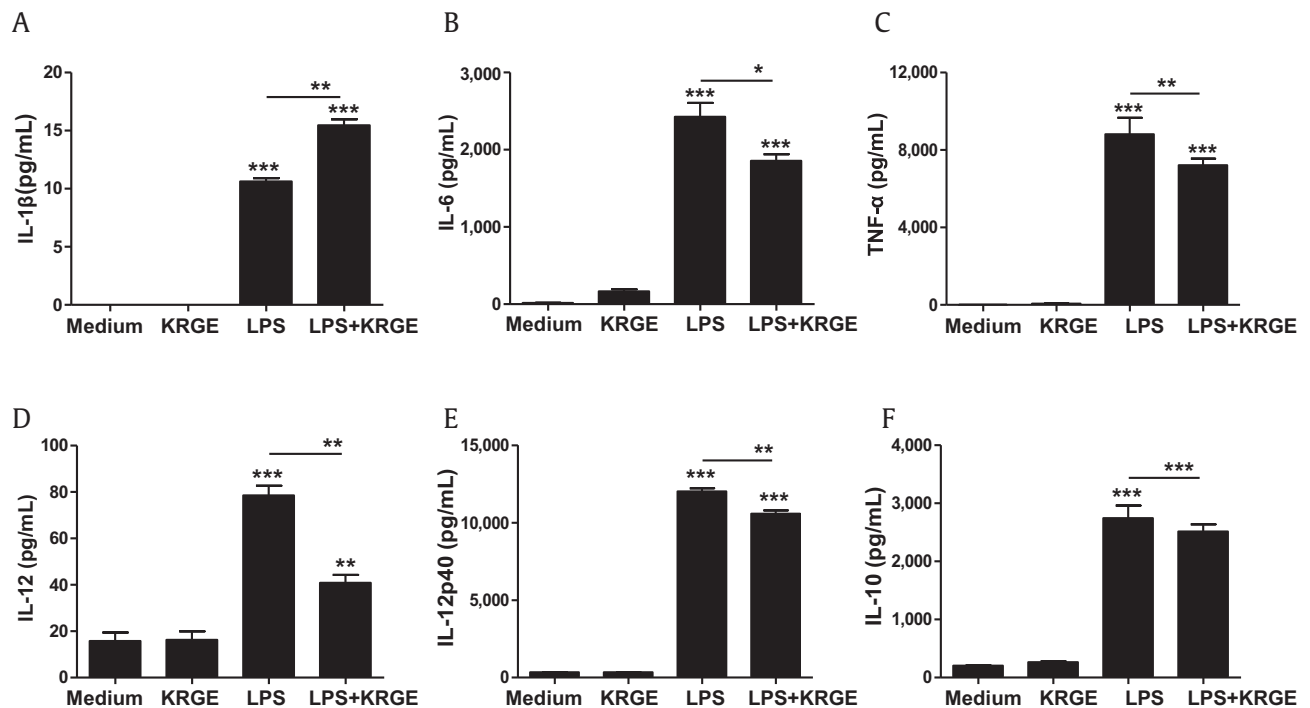


Fig. 1. Function of KRGE on the cytokines production from DCs upon LPS stimulation: The amount of (A) IL-1 β ; (B) IL-6; (C) TNF α ; (D) IL-12; (E) IL-12p40; and (F) IL-10 in DC culture supernatants after stimulation with LPS in the presence or absence of KRGE. Bone marrow DCs were activated with 80 ng/mL of LPS with or without 250 μ g/mL of KRGE for 24 h and the amount of cytokine was quantified by enzyme-linked immunosorbent assay. Experiments were conducted three times. Data represent mean \pm SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 in comparison with medium unless otherwise indicated. DCs, dendritic cells; KRGE, Korean Red Ginseng extract; LPS, lipopolysaccharide; SEM, standard error of the mean.

dextran sodium sulfate- or LPS-induced IL-6, TNF- α , and IL-12p40 from macrophages by downregulating NF κ B [34], it is feasible to surmise that the KRGE-mediated inhibition of IL-6, IL-12p40, and TNF- α from DCs might be also associated with NF κ B activity. KRGE contains different type of saponins (ginsenosides) and non-saponins including ginsan, polysaccharide, protopanaxadiol, and protopanaxatriol [35]. The saponins and non-saponins of ginseng reportedly have opposite effects in IL-1 β production. The saponin fractions of ginseng extract attenuated IL-1 β maturation, whereas non-saponin fractions or water extract of ginseng stimulated IL-1 β production [36,37]. In contrast to these results, the saponin fraction of KRGE has shown to enhance the IL-1 β expression induced by oxazolone. However, the constituent ginsenosides, such as Rf, Rg3, and Rh2, effectively reduced the expression of IL-1 β [38]. These results demonstrate that non-saponins and several types of saponins contained in KRGE could increase the production of IL-1 β from DCs. Of note, we observed that addition of KRGE significantly downregulated the production of IL-12 from DCs induced by LPS (Fig. 1D). IL-12 induces the phosphorylation of signal transducer and activator of transcription 4 (STAT4), leading to the naïve CD4⁺ T cells into the Th1 cell lineage program including induced T-bet expression [39,40]. Since KRGE decreased production of IL-12 from DCs, we hypothesized that KRGE might impact the Th1 cell differentiation.

3.2. KRGE suppresses DC-mediated Th1 cells differentiation

To determine the effect of KRGE on the Th1 cells differentiation, we employed a well-established DC-T cell co-culture *in vitro* system

in which the addition of soluble anti-CD3 and LPS triggers the differentiation of IFN γ -producing Th1 cells from the naïve CD4⁺ T cells [41]. In this DC-T cell system, we observed that the addition of KRGE moderately decreased the frequency of IFN γ -producing Th1 cells (Fig. 2A), likely due to the reduced IL-12 production from DCs. Accordingly, the production of IFN γ from T cells was significantly diminished (Fig. 2B).

To determine if KRGE has any direct effects on T cell during Th1 differentiation, we employed a DC-free Th1 cell differentiation condition by stimulating naïve CD4⁺ T cells. Under this condition, KRGE also exerted a slightly but significant reduction in the frequency of IFN γ ⁺ CD4⁺ T cells and the amount of IFN γ from T cells (Fig. 2C and D), although the magnitude of suppression was weaker than the DC-T cell co-culture system. These results together strongly demonstrate that KRGE inhibits the Th1 cells differentiation *in vitro* by decreasing the IL-12 production and also by directly affecting T cells. Since Th1 cells are well-known as a crucial player for the pathogenesis in autoimmune diseases such as colitis [42] and EAE [43], the use of KRGE might be effective in ameliorating Th1-mediated immune disorders.

3.3. Enhanced Rg3 negatively regulates DC-mediated Th1 cell differentiation

Since Rg3 is a major ginsenoside in KRGE [18,19], we next questioned if enhanced Rg3 plays any role in the KRGE-induced suppression of Th1 cell differentiation. First, we investigated the cell toxicity of enhanced Rg3 on bone marrow DCs, and the MTT reduction assay showed that the concentrations less than 100 μ M

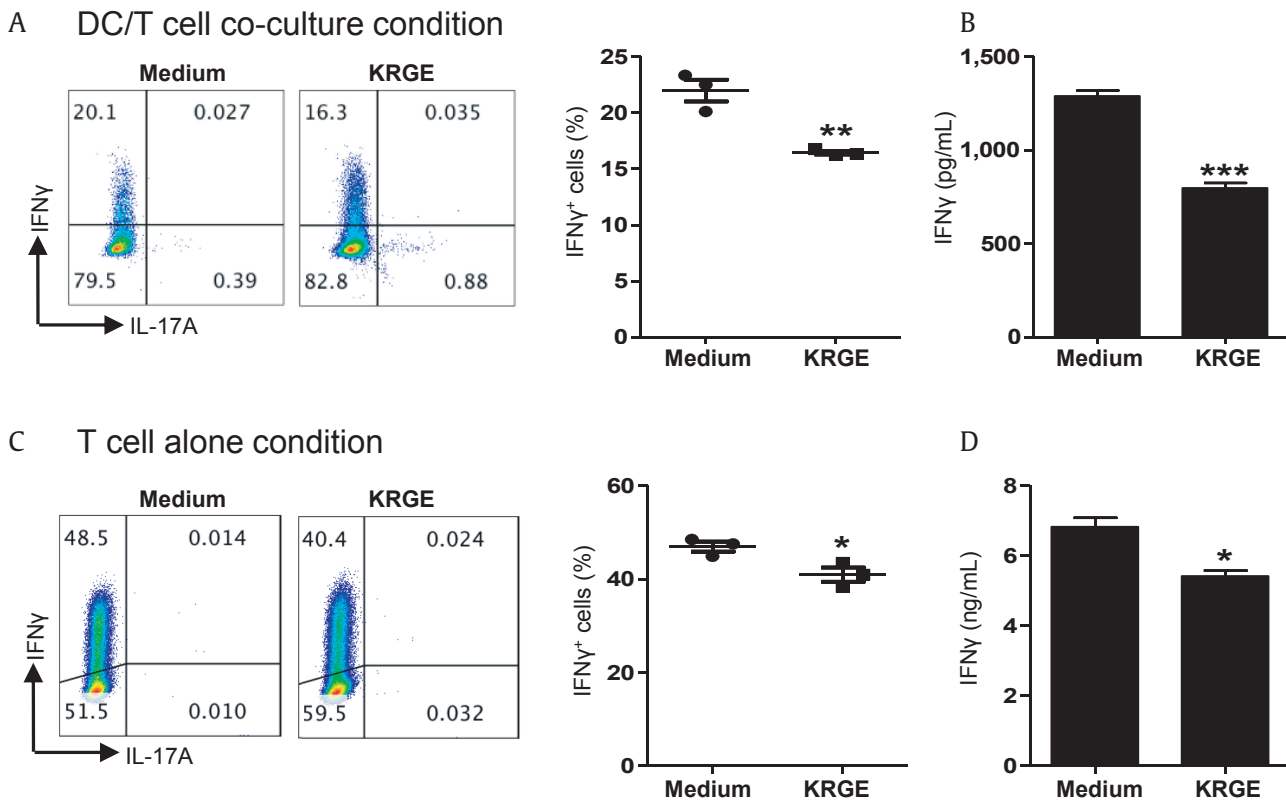


Fig. 2. KRGE inhibits the differentiation of Th1 cell *in vitro* system. To differentiate naïve CD4⁺ T cells (CD4⁺CD62L⁺CD25⁻CD44⁻) into Th1 cells, naïve CD4⁺ T cells were stimulated with LPS in DC-T cell system or with IL-12 and IL-12 in DC-free system in the presence of soluble anti-CD3 or pre-coated anti-CD3 and CD28, respectively. KRGE (250 μ g/mL) was added at the beginning of Th1 cell differentiation. The frequencies of Th1 cells (IFN γ ⁺CD4⁺) (**A and C**) and the quantity of IFN γ in the cell supernatants (**B and D**) were measured by fluorescence-activated cell sorting and enzyme-linked immunosorbent assay, respectively. Data are presented as mean \pm SEM. Data represent one of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. DCs, dendritic cells; KRGE, Korean Red Ginseng extract; LPS, lipopolysaccharide; SEM, standard error of the mean.

have no effect on cell viability (Fig. S1B). We next examined the role of enhanced Rg3 in Th1 cell differentiation. Similar to KRGE, enhanced Rg3 significantly suppressed LPS-induced production of IL-12p70 and IL-6 from DCs (Fig. 3A and Fig. S2A). Moreover, enhanced Rg3 also significantly suppressed the production of IL-12p40 (Fig. 3A), which differed from the effect of KRGE shown in Fig. 1. Unlike KRGE, enhanced Rg3 slightly increased the production of IL-10 (Fig. 3A), which is known to prevent the pathological Th1 cell responses and decrease IL-1 β (Fig. S2B) [44]. Consistent with this observation, addition of enhanced Rg3 significantly reduced DC-mediated Th1 cell differentiation and the production of IFN γ from T cells (Fig. 3B and C). We next examined the effects of enhanced Rg3 on the differentiation of Th1 cell in a DC-free

condition and observed that enhanced Rg3 also significantly suppressed Th1 cell differentiation (Fig. 3D and E).

We next sought to investigate the mechanism of enhanced Rg3-mediated inhibition of Th1 cell differentiation. As depicted in Fig. 4A, the addition of enhanced Rg3 remarkably decreased the frequencies of T-bet $^{+}$ and IFN γ^{+} CD4 $^{+}$ T cells in a dose-dependent manner. The suppression of IFN γ expression by enhanced Rg3 was found to be more sensitive than that of T-bet. In parallel with these protein data, the mRNA expression levels of *Irfg*, *Tbx21*, and *Eomes* (encoding Eomesodermin [45]) were lower in enhanced Rg3-treated T cells in a dose-dependent manner (Fig. 4B). Since STAT4 is a crucial transcription factor for Th1 cell differentiation [46,47], it would be interesting to examine if enhanced Rg3 inhibits

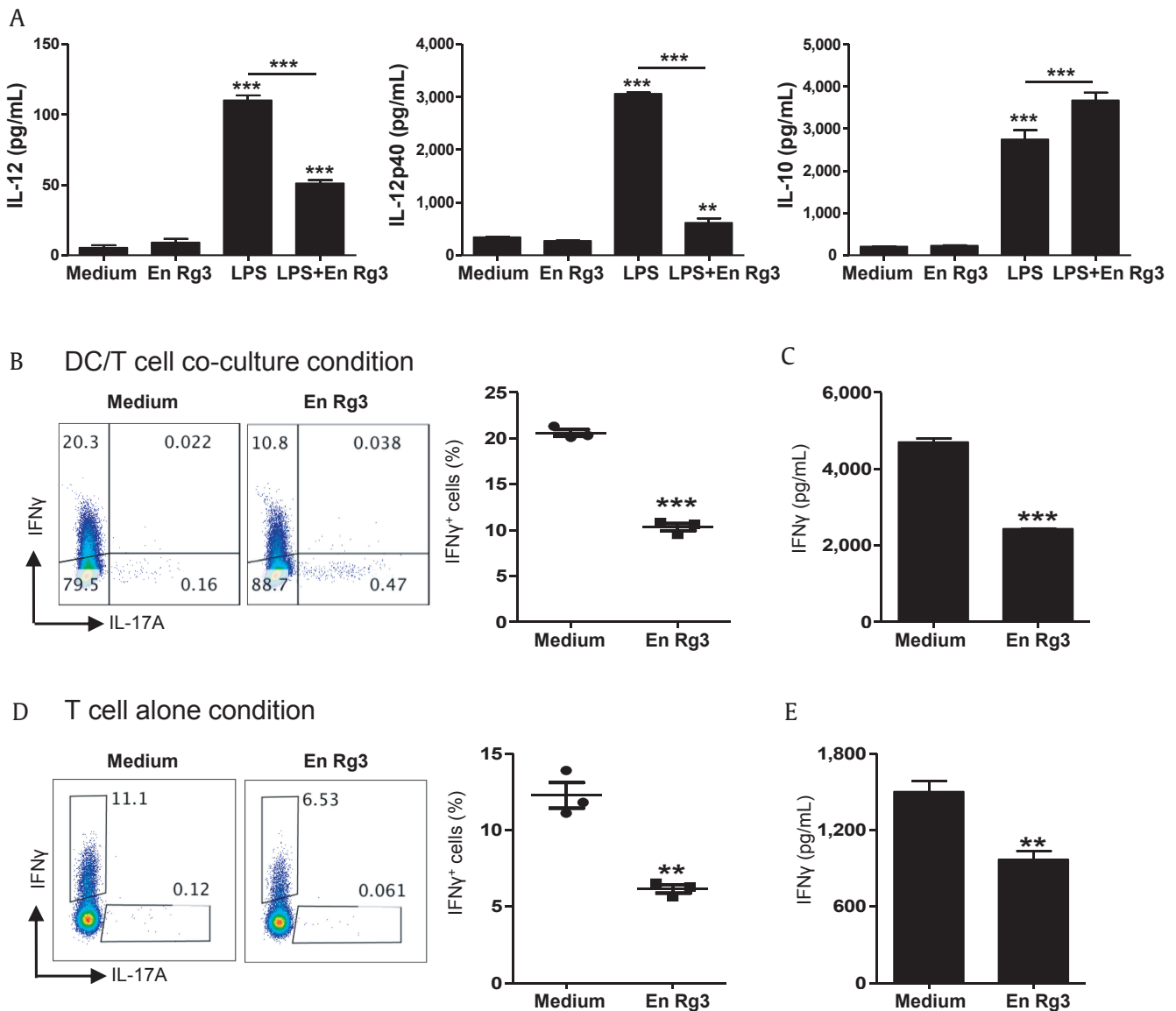


Fig. 3. Enhanced Rg3 suppresses the IL-12 production from DCs and Th1 cell differentiation. (A) The levels of indicated cytokines in bone marrow DC culture supernatants were quantified by enzyme-linked immunosorbent assay after stimulation of bone marrow DC with LPS for 24 h in the presence of 100 μ M enhanced Rg3. Naïve CD4 $^{+}$ T cells and bone marrow DCs were activated by LPS either with enhanced Rg3. Naïve CD4 $^{+}$ T cells were incubated in anti-CD3 and anti-CD28 pre-coated 96-well plate in the stimulation with IL-2 and IL-12 in for Th1 cell development. Enhanced Rg3 (100 μ M) was added at the beginning of Th1 cell differentiation. After 96 h, (B and D) the population of IFN γ^{+} cells gated from CD4 $^{+}$ cells were analyzed by fluorescence-activated cell sorting and (C and E) the level of IFN γ in culture supernatants were evaluated by enzyme-linked immunosorbent assay. (A). (B). (C). (D). (E). Data are mean \pm SEM and represent one experiment from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. DC, dendritic cells; En Rg3, enhanced Rg3; LPS, lipopolysaccharide; SEM, standard error of the mean.

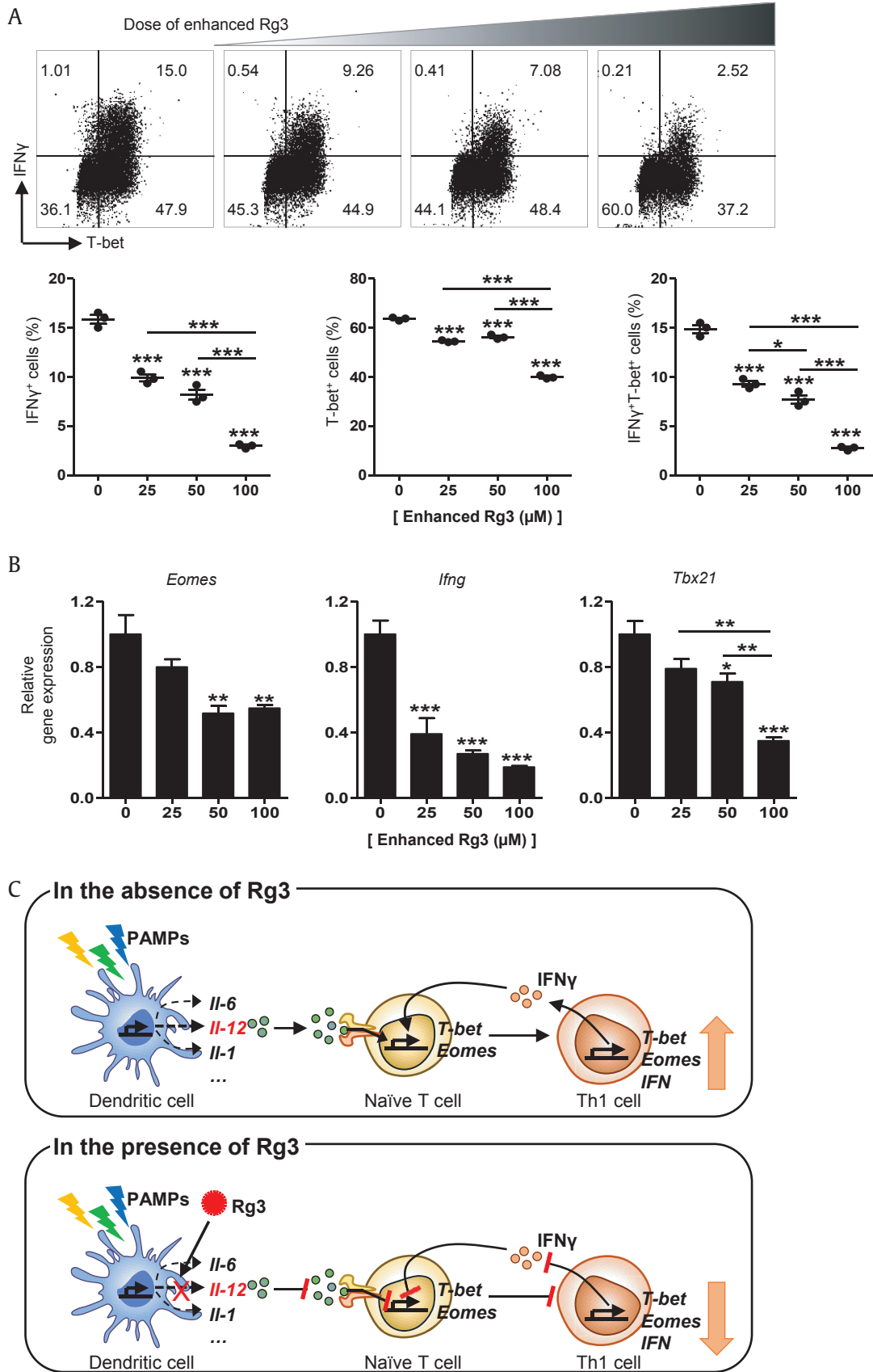
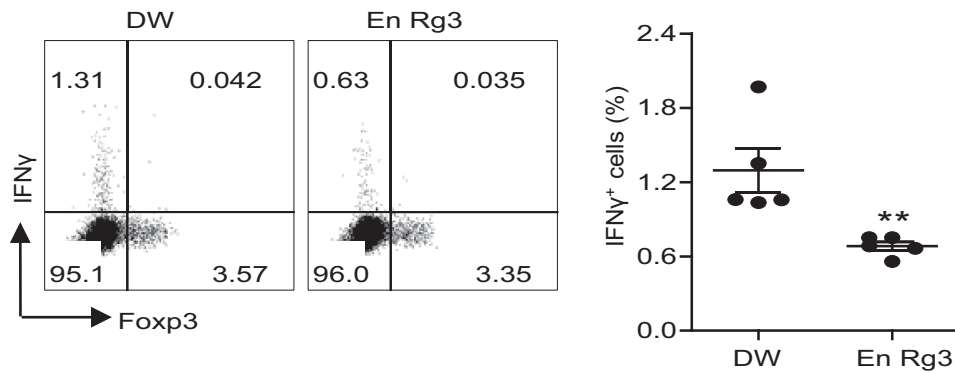
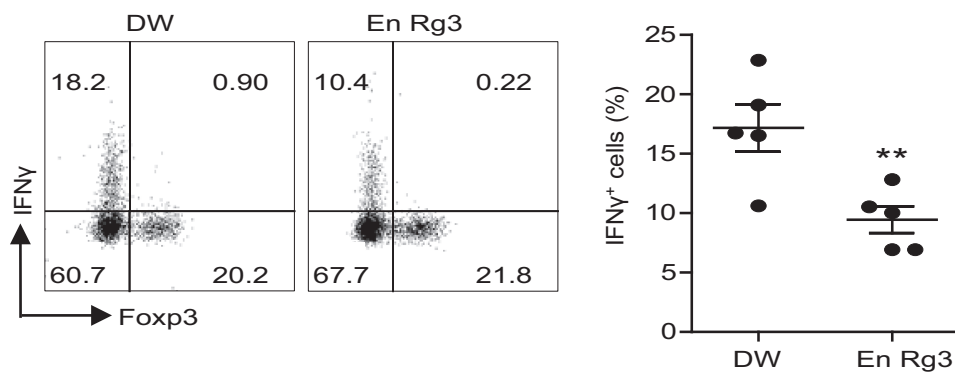


Fig. 4. Regulation of DC-mediated Th1 cell differentiation by enhanced Rg3. Naive CD4⁺ T cells and bone marrow DCs were co-cultured in the presence of LPS and increasing concentrations of enhanced Rg3 for 96 h. **(A)** The frequencies of IFN⁺ and T-bet⁺ CD4⁺ T cells. **(B)** Relative mRNA expression levels were calculated by quantitative real-time polymerase chain reaction. Data represent one of three independent experiments. Data shown are mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 in comparison with 0 μM

A Payer's patch



B Lamina propria



C Mesenteric lymph node

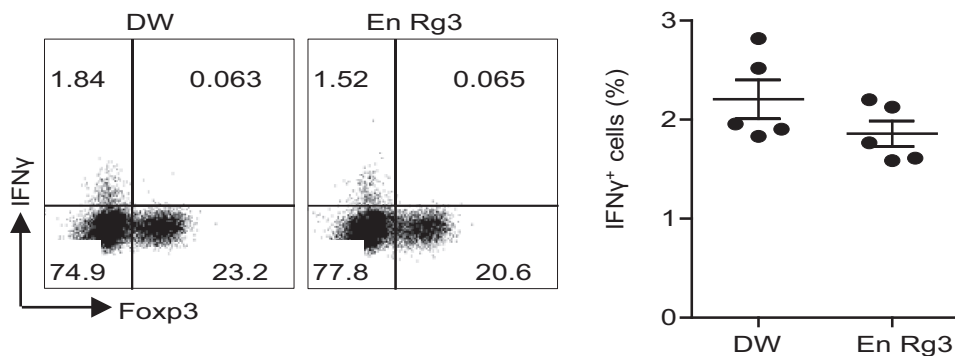


Fig. 5. Oral application of enhanced Rg3 decreases Th1 cell population in the gut. Mice were orally fed with enhanced Rg3 every other day for 4 wk. Flow cytometry results of the frequency of IFN γ ⁺ CD4⁺ T cells in Peyer's patch (A), lamina propria of large intestine (B) and mesenteric lymph nodes (C). Data shown are mean \pm SEM and represent one of two independent experiments. ** $p < 0.01$ versus control group (DW). DW, distilled water; En Rg3, enhanced Rg3; SEM, standard error of the mean.

the phosphorylation of STAT4 in T cells. These findings together indicate that enhanced Rg3 inhibits Th1 cell differentiation via at least two different modes of action by suppressing the production of Th1-promoting IL-12 in DCs and also by inhibiting IL-12-mediated activation of the Th1 cell program in T cells.

3.4. Enhanced Rg3 controls Th1 cell population in the gut

Common microbiota in the intestinal lumen provides high density of PAMPs [48,49], and intestinal CD4⁺ T cell population is an important mediator of immune homeostasis and

unless otherwise indicated. (C) The schematic diagram of the inhibition mechanism of Th1 cell development by enhanced Rg3. Pathogen-associated molecular patterns (PAMPs) on microbes promote DCs to produce pro-inflammatory cytokines. Particularly, in response to IL-12, naive CD4⁺ T cells up-regulate the transcription factors, *T-bet* and *Eomes*, and differentiate into effector Th1 cells which secrete IFN γ . However, in the presence of Rg3, the development of Th1 CD4⁺ T cells is suppressed following decreased IL-12 production by Rg3 in DCs. Enhanced Rg3 also decreased *T-bet*, *Eomes*, and IFN γ through direct interaction with differentiated Th1 CD4⁺ T cells. DC, dendritic cells; LPS, lipopolysaccharide; PAMPs, pathogen-associated molecular patterns; SEM, standard error of the mean.

inflammation [50,51]. Since most of ginseng-related supplements are orally administered, we determined if enhanced Rg3 impacts Th1 cell responses in the gut of normal mice. To this end, we administered enhanced Rg3 or water as a vehicle into groups of naïve C57BL/6 mice and measured the frequency of IFN γ ⁺ CD4⁺ T cells in the gut-associated lymphoid tissues (GALTs) including MLNs, PPs, and lamina propria (LP). As depicted in Fig. 5, we observed the significantly reduced frequency of IFN γ ⁺ CD4⁺ T cells in the PPs and LP in enhanced Rg3-treated mice when compared to that of vehicle-treated mice. The frequency of IFN γ ⁺ CD4⁺ T cells in the MLNs remained unchanged by enhanced Rg3 treatment. On the other hand, the frequency of Foxp3⁺ CD4⁺ T cells appeared to be unaffected by enhanced Rg3 treatment in all the GALTs tested, indicating that there is a little role of enhanced Rg3 on regulatory CD4⁺ T cell population in the gut. Taken together, these results suggest that oral administration of enhanced Rg3 resulted in diminished Th1 cell population in the gut *in vivo*, which is consistent with our observation *in vitro* (Figs. 3 and 4).

It is well documented that T-bet⁺ IFN γ ⁺ CD4⁺ T cells are increased in patients with Crohn's disease (CD) [52,53]. Neutralization of IFN γ as well as deficiency of T-bet significantly ameliorates the induction of experimental colitis in animal models [42]. Moreover, anti-p40 neutralizing antibodies (e.g., ustekinumab and briakinumab) showed a moderate clinical benefit in patients with CD [54–56]. Clinical trial of ustekinumab in Phase 3 moderated the severity of CD who had failed anti-TNF antibody treatment. In addition, anti-p40 antibodies also showed clinical benefits in patients with active CD of Phase 2. These clinical efficacies demonstrate that the blockade of p40 could be a potential therapeutic strategy. Since enhanced Rg3 inhibits the differentiation of Th1 cells as well as the production of IL12p40, our findings suggest that enhanced Rg3 might be effective in ameliorating intestinal inflammatory diseases in humans including CD.

4. Conclusions

In summary, our findings demonstrate that enhanced Rg3 has a profound inhibitory effect on Th1 cell differentiation. This regulatory effect seems to be mediated not only by the inhibition of IL-12 production from DCs but also by directly affecting Th1 cell program in T cells, such as the downregulation of T-bet and eomesodermin. In particular, oral administration of enhanced Rg3 significantly reduced the frequency of Th1 cells in the gut, suggesting that enhanced Rg3 might be effective for the treatment of Th1-mediated inflammatory diseases in the gut.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jgr.2017.08.003>.

References

- [1] Yun TK. Brief introduction of Panax ginseng C.A. Meyer. J Korean Med Sci 2001;16(Suppl):S3–5.
- [2] Baek SH, Bae ON, Park JH. Recent methodology in ginseng analysis. J Ginseng Res 2012;36:119–34.
- [3] Park BG, Jung HJ, Cho YW, Lim HW, Lim CJ. Potentiation of antioxidative and anti-inflammatory properties of cultured wild ginseng root extract through probiotic fermentation. J Pharm Pharmacol 2013;65:457–64.
- [4] Wang L, Yu X, Yang X, Li Y, Yao Y, Lui EM, Ren G. Structural and anti-inflammatory characterization of a novel neutral polysaccharide from North American ginseng (*Panax quinquefolius*). Int J Biol Macromol 2015;74:12–7.
- [5] Park EY, Kim HJ, Kim YK, Park SU, Choi JE, Cha JY, Jun HS. Increase in insulin secretion induced by panax ginseng berry extracts contributes to the amelioration of hyperglycemia in streptozotocin-induced diabetic mice. J Ginseng Res 2012;36:153–60.
- [6] Vuksan V, Sung MK, Sievenpiper JL, Stavro PM, Jenkins AL, Di Buono M, Lee KS, Leiter LA, Nam KY, Arnason JT, et al. Korean red ginseng (*Panax ginseng*) improves glucose and insulin regulation in well-controlled, type 2 diabetes: results of a randomized, double-blind, placebo-controlled study of efficacy and safety. Nutr Metab Cardiovasc Dis 2008;18:46–56.
- [7] Yu C, Wen XD, Zhang Z, Zhang CF, Wu XH, Martin A, Du W, He TC, Wang CZ, Yuan CS. American ginseng attenuates azoxymethane/dextran sodium sulfate-induced colon carcinogenesis in mice. J Ginseng Res 2015;39:14–21.
- [8] Zhang W, Xu L, Cho SY, Min KJ, Oda T, Zhang L, Yu Q, Jin JO. Ginseng berry extract attenuates dextran sodium sulfate-induced acute and chronic colitis. Nutrients 2016;8:199.
- [9] Surh YJ, Ferguson LR. Dietary and medicinal antimutagens and anticarcinogens: molecular mechanisms and chemopreventive potential—highlights of a symposium. Mutat Res 2003;523–524:1–8.
- [10] Jeon BH, Kim CS, Kim HS, Park JB, Nam KY, Chang SJ. Effect of Korean red ginseng on blood pressure and nitric oxide production. Acta Pharmacol Sin 2000;21:1095–100.
- [11] Im EJ, Yayeh T, Park SJ, Kim SH, Goo YK, Hong SB, Son YM, Kim SD, Rhee MH. Antiatherosclerotic effect of Korean red ginseng extract involves regulator of G-protein signaling 5. Evid Based Complement Altern Med 2014;2014:985174.
- [12] Jin SH, Park JK, Nam KY, Park SN, Jung NP. Korean red ginseng saponins with low ratios of protopanaxadiol and protopanaxatriol saponin improve scopolamine-induced learning disability and spatial working memory in mice. J Ethnopharmacol 1999;66:123–9.
- [13] Heo JH, Lee ST, Chu K, Oh MJ, Park HJ, Shim JY, Kim M. An open-label trial of Korean red ginseng as an adjuvant treatment for cognitive impairment in patients with Alzheimer's disease. Eur J Neurol 2008;15:865–8.
- [14] Kaneko H, Nakanishi K. Proof of the mysterious efficacy of ginseng: basic and clinical trials: clinical effects of medical ginseng, Korean red ginseng: specifically, its anti-stress action for prevention of disease. J Pharmacol Sci 2004;95:158–62.
- [15] Kim MK, Lee JW, Lee KY, Yang DC. Microbial conversion of major ginsenoside rb(1) to pharmaceutically active minor ginsenoside rd. J Microbiol 2005;43:456–62.
- [16] Liu WK, Xu SX, Che CT. Anti-proliferative effect of ginseng saponins on human prostate cancer cell line. Life Sci 2000;67:1297–306.
- [17] Lu JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293–302.
- [18] Kwon SW, Han SB, Park IH, Kim JM, Park MK, Park JH. Liquid chromatographic determination of less polar ginsenosides in processed ginseng. J Chromatogr A 2001;921:335–9.
- [19] Lau AJ, Seo BH, Woo SO, Koh HL. High-performance liquid chromatographic method with quantitative comparisons of whole chromatograms of raw and steamed Panax notoginseng. J Chromatogr A 2004;1057:141–9.
- [20] Zhang W, Zhang Y, Ma X, Chen Y. Effects of acupuncture Pishu combined with ginsenoside Rg3 on the immune function of rats with chronic fatigue. Int J Clin Exp Med 2015;8:19022–9.
- [21] Park MW, Ha J, Chung SH. 20(S)-ginsenoside Rg3 enhances glucose-stimulated insulin secretion and activates AMPK. Biol Pharm Bull 2008;31:748–51.
- [22] Sun HY, Lee JH, Han YS, Yoon YM, Yun CW, Kim JH, Song YS, Lee SH. Pivotal roles of ginsenoside Rg3 in tumor apoptosis through regulation of reactive oxygen species. Anticancer Res 2016;36:4647–54.
- [23] Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol 2005;5:296–306.
- [24] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
- [25] Pooley JL, Heath WR, Shortman K. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol 2001;166:5327–30.
- [26] Takei M, Tachikawa E, Hasegawa H, Lee JJ. Dendritic cells maturation promoted by M1 and M4, end products of steroidal ginseng saponins metabolized in digestive tracts, drive a potent Th1 polarization. Biochem Pharmacol 2004;68:441–52.
- [27] Zhang W, Cho SY, Xiang G, Min KJ, Yu Q, Jin JO. Ginseng berry extract promotes maturation of mouse dendritic cells. PLoS One 2015;10, e0130926.

- [28] Kim MH, Byon YY, Ko EJ, Song JY, Yun YS, Shin T, Joo HG. Immunomodulatory activity of ginsan, a polysaccharide of panax ginseng, on dendritic cells. *Korean J Physiol Pharmacol* 2009;13:169–73.
- [29] Su W, Sun AJ, Xu DL, Zhang HQ, Yang L, Yuan LY, Jia JG, Zou YZ, Wu YL, Wang KQ, et al. Inhibiting effects of total saponins of panax ginseng on immune maturation of dendritic cells induced by oxidized-low density lipoprotein. *Cell Immunol* 2010;263:99–104.
- [30] Tung NH, Quang TH, Son JH, Koo JE, Hong HJ, Koh YS, Song GY, Kim YH. Inhibitory effect of ginsenosides from steamed ginseng-leaves and flowers on the LPS-stimulated IL-12 production in bone marrow-derived dendritic cells. *Arch Pharm Res* 2011;34:681–5.
- [31] Yokozawa T, Liu ZW. The role of ginsenoside-Rd in cisplatin-induced acute renal failure. *Ren Fail* 2000;22:115–27.
- [32] Senthil K, Veena V, Mahalakshmi M, Pulla R, Yang DC, Parvatham R. Microbial conversion of major ginsenoside Rb1 to minor ginsenoside Rd by Indian fermented food bacteria. *Afr J Biotechnol* 2009;8:6961–6.
- [33] Stamatou NM, Carubelli I, van de Vlekkert D, Bonten EJ, Papini N, Feng C, Venerando B, d'Azzo A, Cross AS, Wang LX, et al. LPS-induced cytokine production in human dendritic cells is regulated by sialidase activity. *J Leukoc Biol* 2010;88:1227–39.
- [34] Seong MA, Woo JK, Kang JH, Jang YS, Choi S, Jang YS, Lee TH, Jung KH, Kang DK, Hurh BS, et al. Oral administration of fermented wild ginseng ameliorates DSS-induced acute colitis by inhibiting NF-kappaB signaling and protects intestinal epithelial barrier. *BMB Rep* 2015;48:419–25.
- [35] Sathyanath R, Hanumantha Rao BR, Kim HG, Cho JH, Son CG. Saponin and non-saponin fractions of red ginseng ameliorate cisplatin-induced pica in rats. *Pharm Biol* 2013;51:1052–60.
- [36] Shin JY, Song JY, Yun YS, Yang HO, Rhee DK, Pyo S. Immunostimulating effects of acidic polysaccharides extract of Panax ginseng on macrophage function. *Immunopharmacol Immunotoxicol* 2002;24:469–82.
- [37] Han B-C, Ahn H, Lee J, Jeon E, Seo S, Jang KH, Lee S-H, Kim CH, Lee G-S. Nonsaponin fractions of Korean red ginseng extracts prime activation of NLRP3 inflammasome. *J Ginseng Res* 2017;41:513–23.
- [38] Bae EA, Han MJ, Shin YW, Kim DH. Inhibitory effects of Korean red ginseng and its genuine constituents ginsenosides Rg3, Rf, and Rh2 in mouse passive cutaneous anaphylaxis reaction and contact dermatitis models. *Biol Pharm Bull* 2006;29:1862–7.
- [39] Zhou W, Zhang F, Aune TM. Either IL-2 or IL-12 is sufficient to direct Th1 differentiation by nonobese diabetic T cells. *J Immunol* 2003;170:735–40.
- [40] Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993;177:1199–204.
- [41] Jeon YT, Na H, Ryu H, Chung Y. Modulation of dendritic cell activation and subsequent th1 cell polarization by lidocaine. *PLoS One* 2015;10, e0139845.
- [42] Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A, Mizoguchi E, Mudter J, Galle PR, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 2002;195:1129–43.
- [43] Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* 2002;415:536–41.
- [44] Takei M, Tachikawa E, Umeyama A. Dendritic cells promoted by ginseng saponins drive a potent Th1 polarization. *Biomark Insights* 2008;3:269–86.
- [45] Yang Y, Xu J, Niu Y, Bromberg JS, Ding Y. T-bet and eomesodermin play critical roles in directing T cell differentiation to Th1 versus Th17. *J Immunol* 2008;181:8700–10.
- [46] Good SR, Thieu VT, Mathur AN, Yu Q, Stritesky GL, Yeh N, O'Malley JT, Perumal NB, Kaplan MH. Temporal induction pattern of STAT4 target genes defines potential for Th1 lineage-specific programming. *J Immunol* 2009;183:3839–47.
- [47] O'Shea JJ, Plenge R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. *Immunity* 2012;36:542–50.
- [48] Santaolalla R, Fukata M, Abreu MT. Innate immunity in the small intestine. *Curr Opin Gastroenterol* 2011;27:125–31.
- [49] Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nat Immunol* 2013;14:668–75.
- [50] Shale M, Schiering C, Powrie F. CD4(+) T-cell subsets in intestinal inflammation. *Immunol Rev* 2013;252:164–82.
- [51] van Wijk F, Cheroutre H. Mucosal T cells in gut homeostasis and inflammation. *Expert Rev Clin Immunol* 2010;6:559–66.
- [52] Frisullo G, Nociti V, Iorio R, Patanella AK, Plantone D, Bianco A, Marti A, Cammarota G, Tonali PA, Batocchi AP. T-bet and pSTAT-1 expression in PBMC from coeliac disease patients: new markers of disease activity. *Clin Exp Immunol* 2009;158:106–14.
- [53] Monteleone I, Monteleone G, Del Vecchio Blanco G, Vavassori P, Cucchiara S, MacDonald TT, Pallone F. Regulation of the T helper cell type 1 transcription factor T-bet in coeliac disease mucosa. *Gut* 2004;53:1090–5.
- [54] Mannon PJ, Fuss JJ, Mayer L, Elson CO, Sandborn WJ, Present D, Dolin B, Goodman N, Groden C, Hornung RL, et al. Anti-interleukin-12 antibody for active Crohn's disease. *N Eng J Med* 2004;351:2069–79.
- [55] Sandborn WJ, Feagan BG, Fedorak RN, Scherl E, Fleisher MR, Katz S, Johanss J, Blank M, Rutgeerts P, Grp UCDS. A randomized trial of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology* 2008;135:1130–41.
- [56] Fuss JJ, Becker C, Yang ZQ, Groden C, Hornung RL, Heller F, Neurath MF, Strober W, Mannon PJ. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 2006;12:9–15.