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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 (IFNγ) 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.

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 (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 proinflammatory 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 TNFα, and ginseng-primed DCs improved the CD4$^+$ T cell proliferations and the interferon gamma

(IFNγ) 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 IFNγ$^+$ 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 μg/mL, 31.25 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, and 500 μg/mL) and enhanced Rg3 (0 μM, 25 μM, 50 μM, 100 μM, and 200 μM) were treated with bone marrow-derived DCs for 24 h; the cells were then incubated with MTT solution (final conc. 500 μg/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 μM 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-CD3ε (0.3 μg/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-CD3ε (1 μg/mL) and anti-CD28 (2 μg/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 μg/mL) or various concentration of enhanced Rg3 (25 μM, 50 μM, and 100 μM) were treated at the beginning of naïve CD4$^+$ T cells.

2.7. Enzyme-linked immunosorbent assay

IL-1β, 6, 12p70, 12p40, and TNFα 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 Cosmogenetech (Seoul, South Korea): *Ifn* (sense, 5′-GATGCATTCATGAGTATTGCCAAGT-3′, antisense, 5′-GTGGACCACTCGGATGAGCTC-3′), *Eomes* (sense, 5′-TGAATGAACCTTCCAAGACTCAGA-3′, antisense, 5′-GGCTTGAGGCAAAGTGTTGACA-3′), *T-bet* (sense, 5′-CAACAACCCCTTTGCCAAAG-3′, antisense, 5′-TCCCCCAAGCAGTTGACAGT-3′), *Gata3* (sense, 5′-AGAACCGGCCCCTTATGAA-3′, antisense, 5′-AGTTCGCGCAGGATGTCC-3′), *Rorc* (sense, 5′-CCGCTGAGAGGGCTTCAC-3′, antisense, 5′-TGCAGGATAGGCCACATTACA-3′), *Gzmb* (sense, 5′-GCCCACAACATCAAAGAACAG-3′, antisense, 5′-AACCAGCCACATAGCACACAT-3′), *β-actin* (sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′).

2.9. Flow cytometry

Cells were incubated for 3–4 h with 100 ng/mL of PMA, 1 μM 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-IFNγ 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 μm-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 μg/mL (Fig. S1A). On the basis of this result, we used KRGE at the concentration of 250 μg/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-1β, IL-6, IL-12, IL-23, and TNFα [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 TNFα induced by LPS (Fig. 1B–F). By contrast, the production of LPS-induced IL-1β 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
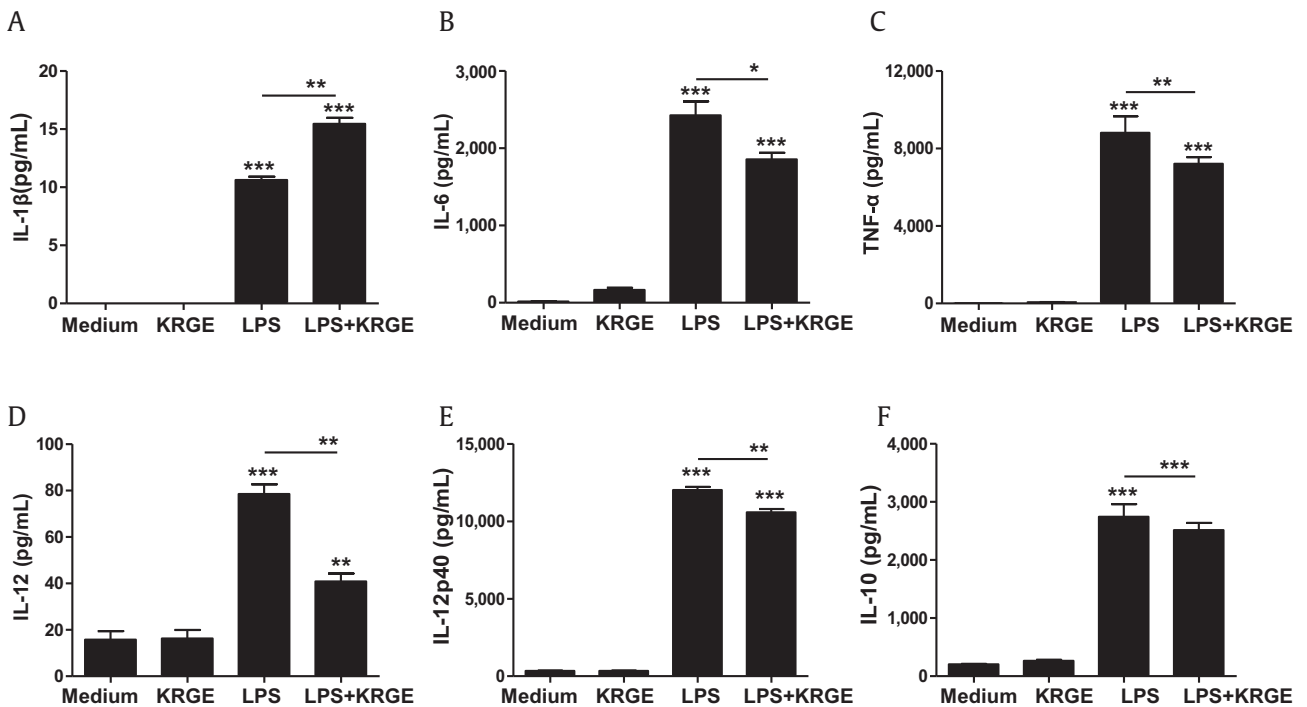

Fig. 1. Function of KRGE on the cytokines production from DCs upon LPS stimulation: The amount of **(A)** IL-1β; **(B)** IL-6; **(C)** TNFα; **(D)** IL-12; **(E)** IL-12p40; and **(F)** IL-10 in DC culture supernatants after stimulation with LPS in the presence or absence of KRGE. Bone marrow DCs were activated with 80 ng/mL of LPS with or without 250 μg/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 NFκB [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 NFκB 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-1β production. The saponin fractions of ginseng extract attenuated IL-1β maturation, whereas non-saponin fractions or water extract of ginseng stimulated IL-1β production [36,37]. In contrast to these results, the saponin fraction of KRGE has shown to enhance the IL-1β expression induced by oxazolone. However, the constituent ginsenosides, such as Rf, Rg3, and Rh2, effectively reduced the expression of IL-1β [38]. These results demonstrate that non-saponins and several types of saponins contained in KRGE could increase the production of IL-1β 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 IFNγ-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 IFNγ-producing Th1 cells (Fig. 2A), likely due to the reduced IL-12 production from DCs. Accordingly, the production of IFNγ 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 IFNγ$^+$ CD4$^+$ T cells and the amount of IFNγ 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 gensenoside 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 100μM
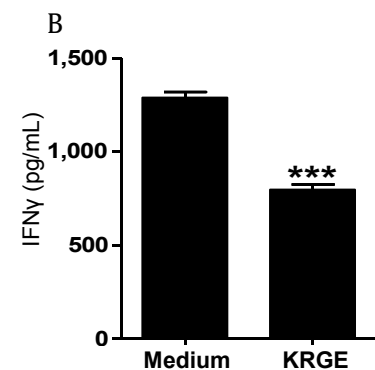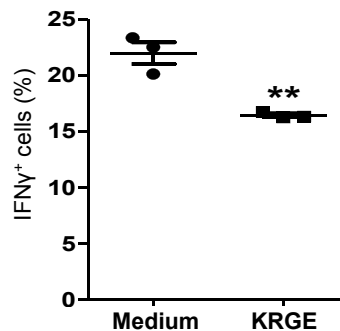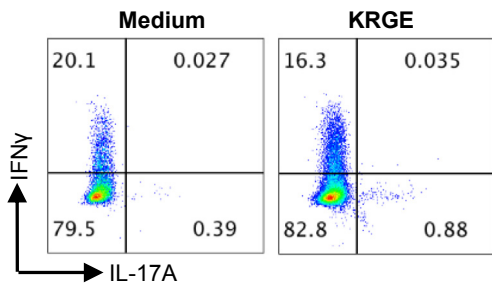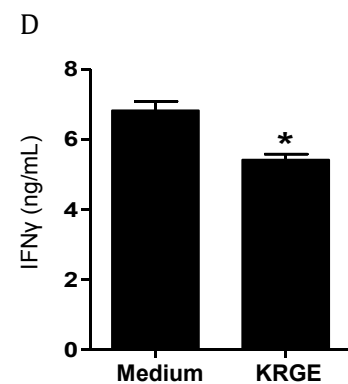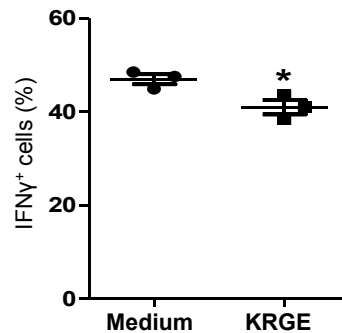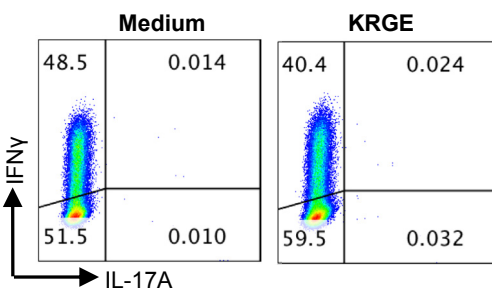

Fig. 2. . KRGE inhibits the differentiation of Th1 cell *in vitro* system. To differentiate naïve CD4$^+$ T cells (CD4$^+$CD62L$^+$CD25$^-$CD44$^-$) into Th1 cells, naïve CD4$^+$ T cells were stimulated with LPS in DC-T cell system or with IL-12 and IL-12 in DC-free system in the presence of soluble anti-CD3 or pre-coated anti-CD3 and CD28, respectively. KRGE (250 μg/mL) was added at the beginning of Th1 cell differentiation. The frequencies of Th1 cells (IFNγ$^+$CD4$^+$) (**A and C**) and the quantity of IFNγ 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 ± 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-1β (Fig. S2B) [44]. Consistent with this observation, addition of enhanced Rg3 significantly reduced DC-mediated Th1 cell differentiation and the production of IFNγ 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 IFNγ+ CD4+ T cells in a dose-dependent manner. The suppression of IFNγ 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 *Ifng*, *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
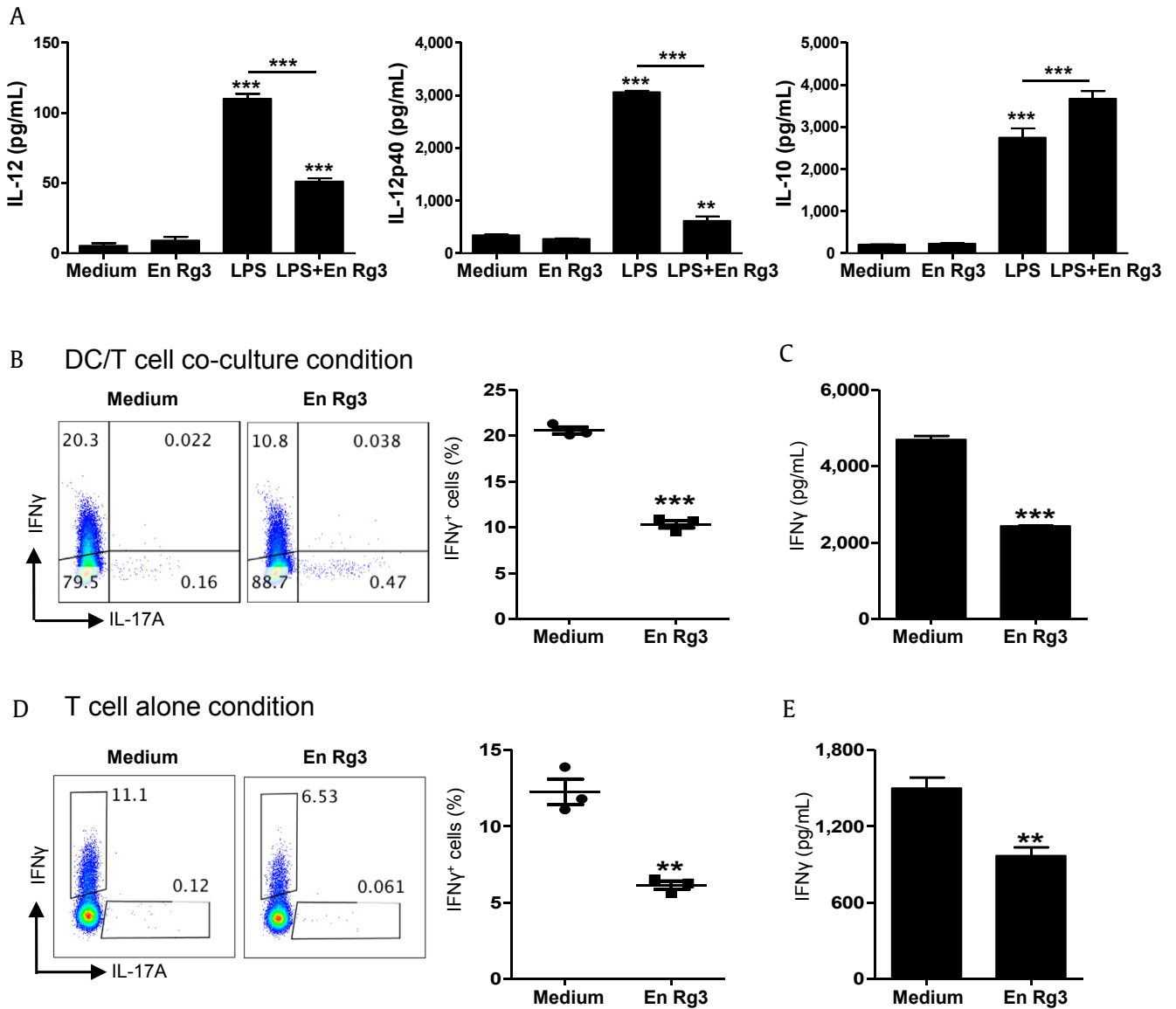

Fig. 3. Enhanced Rg3 suppresses the IL-12 production from DCs and Th1 cell differentiation. **(A)** The levels of indicated cytokines in bone marrow DC culture supernatants were quantified by enzyme-linked immunosorbent assay after stimulation of bone marrow DC with LPS for 24 h in the presence of 100 μM 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 μM) was added at the beginning of Th1 cell differentiation. After 96 h, **(B and D)** the population of IFNγ+ cells gated from CD4+ cells were analyzed by fluorescence-activated cell sorting and **(C and E)** the level of IFNγ in culture supernatants were evaluated by enzyme-linked immunosorbent assay. (A) . (B) . (C) . (D) . (E) . Data are mean ± 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.
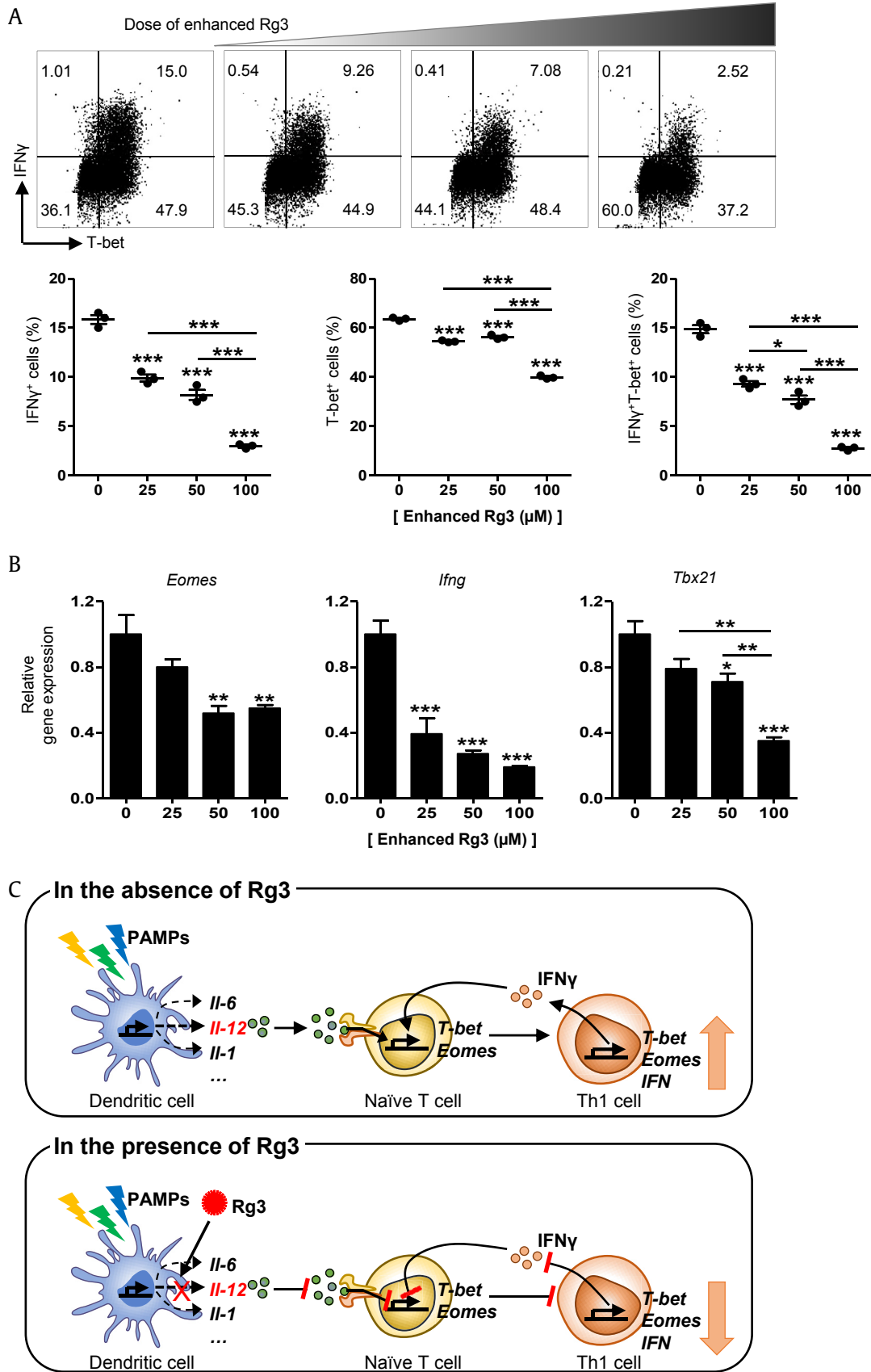
Fig. 4. Regulation of DC-mediated Th1 cell differentiation by enhanced Rg3. Naïve CD4^{+} T cells and bone marrow DCs were co-cultured in the presence of LPS and increasing concentrations of enhanced Rg3 for 96 h. **(A)** The frequencies of IFNγ^{+} and T-bet^{+} CD4^{+} T cells. **(B)** Relative mRNA 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 0μM
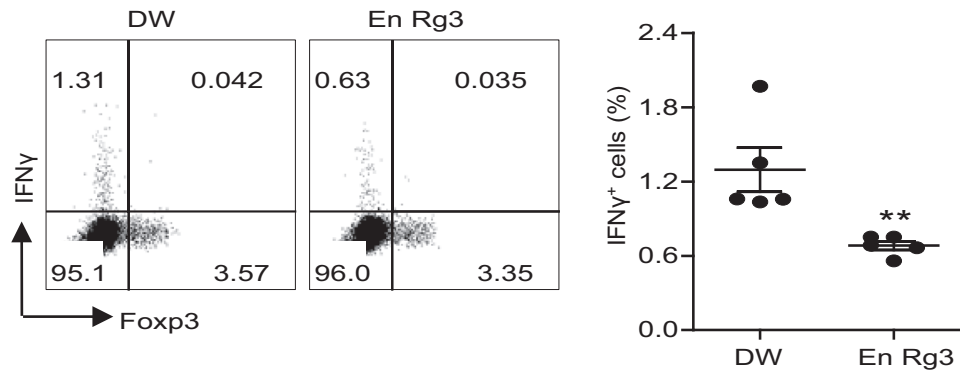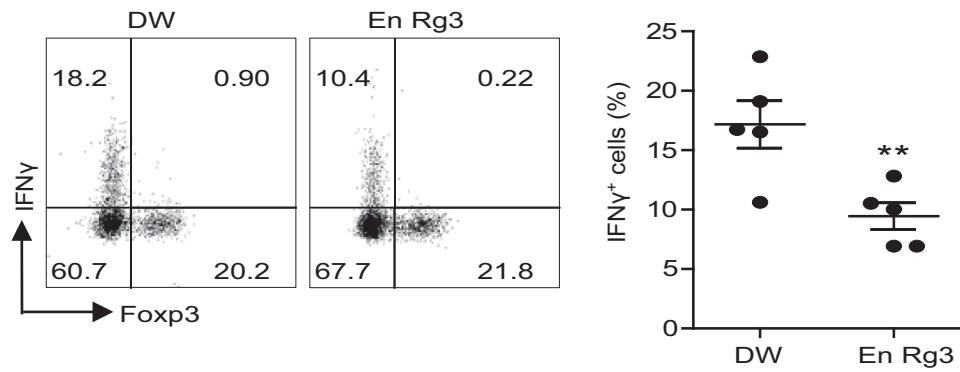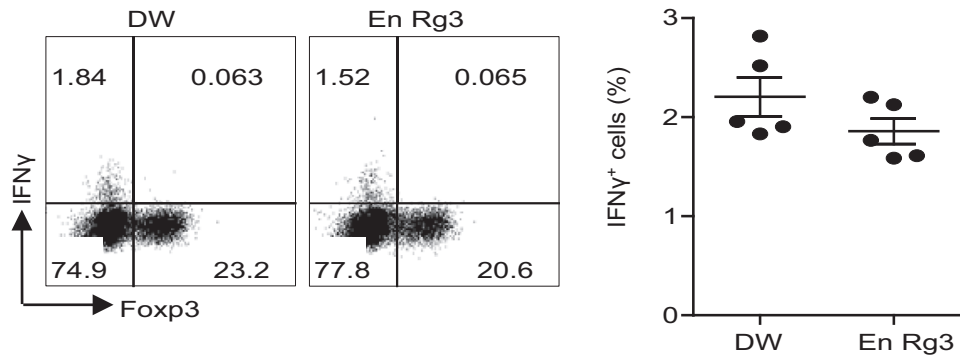
Fig. 5. Oral application of enhanced Rg3 decreases Th1 cell population in the gut. Mice were orally fed with enhanced Rg3 every other day for 4 wk. Flow cytometry results of the frequency of IFNγ+ CD4+ T cells in Peyer's patch (**A**), lamina propria of large intestine (**B**) and mesenteric lymph nodes (**C**). Data shown are mean ± 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, naïve CD4+ T cells up-regulate the transcription factors, *T-bet* and *Eomes*, and differentiate into effector Th1 cells which secrete IFNγ. 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 *IFNγ* 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 IFNγ^+ 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 IFNγ^+ CD4$^+$ T cells in the PPs and LP in enhanced Rg3-treated mice when compared to that of vehicle-treated mice. The frequency of IFNγ^+ 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$^+$ IFNγ^+ CD4$^+$ T cells are increased in patients with Crohn's disease (CD) [52,53]. Neutralization of IFNγ 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.

Acknowledgments

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.

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