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# T Cell Stimulatory Effects of Korean Red Ginseng through Modulation of Myeloid-Derived Suppressor Cells

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Myeloid-derived suppressor cells (MDSCs) actively suppress immune cells and have been considered as an impediment to successful cancer immunotherapy. Many approaches have been made to overcome such immunosuppressive factors and to exert effective anti-tumor effects, but the possibility of using medicinal plants for this purpose has been overlooked. Korean red ginseng (KRG) is widely known to possess a variety of pharmacological properties, including immunoboosting and anti-tumor activities. However, little has been done to assess the anti-tumor activity of KRG on MDSCs. Therefore, we examined the effects of KRG on MDSCs in tumor-bearing mice and evaluated immunostimulatory and anti-tumor activities of KRG through MDSC modulation. The data show that intraperitoneal administration of KRG compromises MDSC function and induces T cell proliferation and the secretion of IL-2 and IFN-γ, while it does not exhibit direct cytotoxicity on tumor cells and reduced MDSC accumulation. MDSCs isolated from KRG-treated mice also express significantly lower levels of inducible nitric oxide synthase and IL-10 accompanied by a decrease in nitric oxide production compared with control. Taken together, the present study demonstrates that KRG enhances T cell function by inhibiting the immunosuppressive activity of MDSCs and suggests that although KRG alone does not exhibit direct anti-tumor effects, the use of KRG together with conventional chemo- or immunotherapy may provide better outcomes to cancer patients through MDSC modulation.

**Keywords:** *Panax ginseng,* Korean red ginseng, Myeloid-derived suppressor cells, T-lymphocyte activation, Immunomodulation

## INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population composed of myeloid progenitor cells and immature myeloid cells (IMCs) [1]. In normal conditions, IMCs rapidly differentiate and mature into functional granulocytes, macrophages, or dendritic cells. However, in some disease states, such as cancer, infection, transplantation, and autoimmune disease, a partial block occurs in certain stages of myeloid differentiation, leading to the abnormal expansion and accumulation of MDSCs in the bone marrow, spleen, lymph nodes, peripheral blood, and tumor sites [2-6]. In mice, MDSCs are defined by the expression of the granulocyte differentiation antigen Gr1 (or Ly6G) and CD11b (α1βM integrin) [7] and can be further subdivided into $CD11b^+Ly6G^{lo}Ly6C^{hi}$ monocytic MDSCs and $CD11b^+Ly6G^{hi}Ly6C^{lo}$ granulocytic MDSCs [8,9].

Functionally, MDSCs have been reported to suppress immune responses by affecting both innate and acquired immunity. In particular, MDSCs strongly perturb T cell proliferation and T cell activation and are thereby considered to mediate tumor-induced immune dysfunction in patients with tumor burdens [1]. Studies have shown that

the arginine-metabolizing enzyme arginase I is highly activated in MDSCs, and the depletion of arginine in MDSCs results in the inhibition of T cell proliferation [10,11]. In addition, MDSCs up-regulate the expression of inducible nitric oxide synthase (iNOS) which produces T cell suppressive nitric oxide (NO) [12-14]. Reactive oxygen species (ROS) and, in particular peroxynitrite, can also induce post-translational modification of T cell receptors and may cause T cell unresponsiveness [7,15,16]. Given that MDSCs are one of the immunosuppressive factors in cancer and some other diseases, modulation of MDSCs might be a good therapeutic strategy to overcome MDSC-induced immune dysfunction [17-20].

Korean red ginseng (KRG) is heat-processed *Panax ginseng* which has gone through steaming and drying processes to enhance pharmacological activities and stability [21]. As an important traditional medicinal herb in East Asian countries including Korea, it has been shown to possess beneficial effects on immune function, insulin resistance, cancer, hypertension, neurodegenerative disorders, and stress [21-28]. In particular, the anti-tumor activity of KRG has been extensively examined in a variety of cancer models including an epidemiological study [29], and its protective mechanisms have been reported to include an increase in apoptosis, decrease of cell proliferation and telomerase activity, and inhibition of P-glycoprotein [30-34]. However, little has been done to assess the anti-tumor activity of KRG on MDSCs.

Therefore, we examined the effects of KRG on MDSCs in tumor-bearing mice and evaluated the immunomodulatory and anti-tumor activities of KRG. The present study demonstrates that KRG extract enhances T cell proliferation and T cell activation by inhibiting the immunosuppressive activity of MDSCs, although KRG does not directly affect tumor growth.

## MATERIALS AND METHODS

### Korean red ginseng extract
KRG water extract was provided by Korea Ginseng Corporation (Seoul, Korea). The KRG extract was dissolved to 20 mg/mL in sterilized phosphate-buffered saline (PBS).

### Mice and experimental design
Six week-old male C57BL/6 mice were purchased from Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea) and were acclimated for 7 d before experiments. The mice were randomly divided into a control group ($n$=5) and a KRG group ($n$=6), and daily received intraperitoneal injection of PBS or KRG (100 mg/kg), respectively. Two wk later, $5 \times 10^5$ EL-4 thymoma cells suspended in 100 μL PBS were injected subcutaneously into the flank of mice to induce tumors and splenic MDSC accumulation. After an additional 4 wk daily administration of PBS or KRG, mice were sacrificed for splenic MDSC isolation and analysis. Tumor weights were also measured, and tumor size was expressed as the product of perpendicular diameters of individual tumors. All animal experiments were approved by the Animal Care and Use Committee of Chung-Ang University.

### Cell culture
EL-4 thymoma cells, primary MDSCs, and primary CD4$^+$ T cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. For T cell proliferation and cytokine production, T cells were stimulated with anti-CD3 antibody (1 μg/mL; clone 145-2C11, eBioscience, Carlsbad, CA, USA) and anti-CD28 antibody (1 μg/mL; clone 37.51, eBioscience) for 4 d. The cells were cultured at 37 °C in a humidified 5% CO$_2$ atmosphere.

### Isolation of myeloid-derived suppressor cells and CD4$^+$ T cells
Total MDSCs were purified from splenocytes by magnetic cell sorting (MACS; Miltenyi Biotec, Auburn, CA, USA) using anti-CD11b microbeads (Miltenyi Biotec) following the instructions of the manufacturer. In tumor-bearing mice, approximately 95% of CD11b$^+$ splenocytes were Gr1$^+$, and MDSC purity after MACS separation was typically above 85%. For isolation of CD4$^+$ T cells, CD4$^+$ splenocytes were positively selected using anti-CD4 microbeads (Miltenyi Biotec); the purity for CD4$^+$ T cells was greater than 95%.

### Flow cytometric analysis
Spleens were harvested under sterile conditions. Single cell suspensions were prepared, and red blood cells were removed using ACK lysis buffer (NH$_4$Cl, 8.29 g/L; KHCO$_3$, 1.00 g/L; and Na$_2$EDTA·2H$_2$O, 0.0372 g/L). Splenocytes were then stained with fluorochrome-conjugated monoclonal antibodies (clones shown in parentheses) against mouse Gr1 (Ly6G; clone RB6-8C5, BD Biosciences, San Diego, CA, USA), Ly6C (clone HK1.4, BD Biosciences), CD11b (clone M1/70, eBioscience), and CD4 (clone RM4-5, eBioscience). Flow cytometric separations were performed using a FACSCalibur (BD Biosciences), and data analyses were performed using

BD CellQuest Pro software. Granulocytic MDSCs were identified according to the CD11b$^+$Ly6G$^{hi}$Ly6C$^{lo}$ phenotype, and monocytic MDSCs were defined based on their CD11b$^+$Ly6G$^{lo}$Ly6C$^{hi}$ phenotype as described previously [8,9].

### T cell proliferation assay

T cell proliferation was assessed by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). CD4$^+$ T cells isolated from normal C57BL/6 mice and MDSCs were seeded into a 96-well plate at $2 \times 10^4$ and $1 \times 10^4$ cells/well, respectively, coated with anti-CD3 and anti-CD28 antibodies in 200 μL medium. The cells were co-cultured for 4 d, and WST-8 solution containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt was added to a final concentration of 10% (v/v) in each well. The converted orange product, formazan, was colorimetrically measured at 450 nm using a Genius Pro EIS plate reader (Tecan, Mannedorf, Switzerland).

### Measurement of IL-2 and IFN-γ

CD4$^+$ T cells and MDSCs were seeded at the densities detailed above into a 96-well plate coated with anti-CD3 and anti-CD28 antibodies and co-cultured for 4 d. Culture supernatants were then harvested, and the quantity of cytokines was assessed by sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, the microplate was coated with the primary capture antibody diluted to a concentration of 2 μg/mL in coating buffer (0.1 M NaH-CO$_3$). After washing and blocking with PBS containing 3% bovine serum albumin, the plate was incubated with the culture supernatants overnight. The plate was then incubated with biotinylated secondary antibody for 1 h, and streptavidin-alkaline phosphatase (BD Biosciences) was added to the plate. After a 30 minute-incubation, the phosphatase substrate *P*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) was added, and the optical density of each well was determined using a microplate reader at 405 nm. Purified anti-IL-2 (clone JES6-1A12, BD Biosciences) and anti-IFN-γ (clone R4-6A2, BD Biosciences) antibodies were used as the primary antibodies, and biotinylated anti-IL-2 (clone JES6-5H4, BD Biosciences) and anti-IFN-γ (clone XMG1.2, eBioscience) antibodies were used as the secondary antibodies. Recombinant murine IL-2 (eBioscience) and IFN-γ (BD Biosciences) were used as standards to quantify the amount of cytokines produced from the culture. Standards and samples were assayed in triplicate.

### RNA isolation and real-time PCR

MDSCs were seeded into 24-well plates at $1 \times 10^6$ cells/well in 1 mL medium and stimulated with lipopolysaccharide (5 μg/mL; Sigma-Aldrich) for 24 h [35]. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The cDNA was then used for PCR with 2x iQ$^{TM}$ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) to determine the mRNA level of arginase, IL-10, iNOS, and GAPDH. Amplification was performed under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s, followed by a final extension of 55°C for 1 min using an iQ5 Thermal Cycler (Bio-Rad). Gene expression was normalized to the expression of GAPDH. For PCRs, the following primer sets were used: arginase sense, 5'-CAGAGTAT-GACGTGAGAGACCAC-3' and arginase antisense, 5'-CAGCTTGTCTACTTCAGTCATGGAG-3'; IL-10 sense, 5'-CTCTTACTGACTGGCATGAGG-3' and IL-10 antisense, 5'-CCTTGTAGACACCTTG-GTCTTGGAG-3'; iNOS sense, 5'-AGACGGATAG-GCAGAGATTGG-3' and iNOS antisense, 5'-ACT-GACACTTCGGACAAAGC-3'; and GAPDH sense, 5'-AAT GGTGAAGGTCGGTGTGAAC-3' and GAPDH antisense, 5'-GAAGATGGTGATGGGCTTCC-3'.

### Measurement of nitric oxide

The generation of NO was determined by measuring nitrite, a stable final product of NO accumulated in culture media, using Griess reagent. MDSCs were stimulated with LPS for 24 h, and the culture supernatant was collected. The supernatant was then mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H$_3$PO$_4$, Sigma-Aldrich) and incubated for 5 min at room temperature. The absorbance was measured at 540 nm using a ThermoMax Plate Reader (Molecular Devices, Sunnyvale, CA, USA), and the amount of NO was quantified based on a standard curve prepared using sodium nitrite (Junsei Chemical Co., Ltd., Chou-Ku, Japan).

### Statistical analysis

All data are expressed as mean±standard deviation, and the Mann-Whitney *U*-test or Student's *t*-test was used for data analysis. Values were considered statistically significant with *p*<0.05. Absence of significance was not reported for brevity ($^*p$<0.05, $^{**}p$<0.01, and $^{***}p$<0.001).
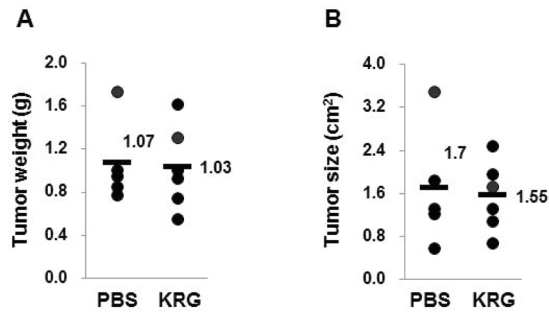
**Fig. 1.** Comparison of tumor weight (A) and size (B) in mice treated with Korean red ginseng (KRG) or phosphate-buffered saline (PBS). Tumor size was expressed as the product of perpendicular diameters of individual tumors. Each circle represents data from an individual mouse, and the solid lines indicate means for each group. Data are presented as the mean±standard deviation for *n*=5 PBS-treated and *n*=6 KRG-treated animals. The data are representative of four experiments with similar results.

## RESULTS

### Korean red ginseng does not affect tumor growth directly

To evaluate the effect of KRG on tumor growth, we injected EL-4 tumor cells into the flank of mice subcutaneously and observed tumor growth. KRG extract was daily administered intraperitoneally for 2 wk prior to tumor implantation and for another 4 wk post tumor challenge. Four weeks after tumor implantation, mice bearing EL-4 tumors were sacrificed, and tumors were removed and weighed. As shown in Fig. 1A and 1B, the tumor weights and sizes of the KRG group are not significantly different from those of the placebo group, indicating that KRG does not directly suppress tumor growth.

### Korean red ginseng does not change myeloid-derived suppressor cell phenotype and accumulation

We then asked whether KRG treatment changed the accumulation of CD11b$^+$Gr1$^+$ MDSCs in the spleen. In contrast to the 2% to 4% seen in normal mice, as many as 20% to 40% of nucleated splenocytes are CD11b$^+$Gr1$^+$ MDSCs in tumor-bearing mice. Recent studies have shown that heterogeneous MDSCs can be separated into two subsets: CD11b$^+$Ly6G$^{hi}$Ly6C$^{lo}$ granulocytic MDSCs and CD11b$^+$Ly6G$^{lo}$Ly6C$^{hi}$ monocytic MDSCs. The two subsets possess different functions and diverse mechanisms for the suppression of T cell function, and in most cases, granulocytic MDSCs expand much faster than the monocyte subset. Splenocytes were isolated from KRG or PBS-treated tumor-bearing mice and subjected to flow cytometric analysis. Our data demonstrated that the percentage and absolute number of splenic CD11b$^+$Gr1$^+$ (equal to CD11b$^+$Ly6G$^+$) MDSCs isolated from the KRG-treated group are not significantly different compared to those isolated from the placebo group (Fig. 2A, B). Further fractionation of MDSCs using the Ly6C monocyte marker separated granulocytic MDSCs from monocytic MDSCs, but the frequencies of granulocytic MDSCs and monocytic MDSCs were not altered by KRG administration (Fig. 2C, D).
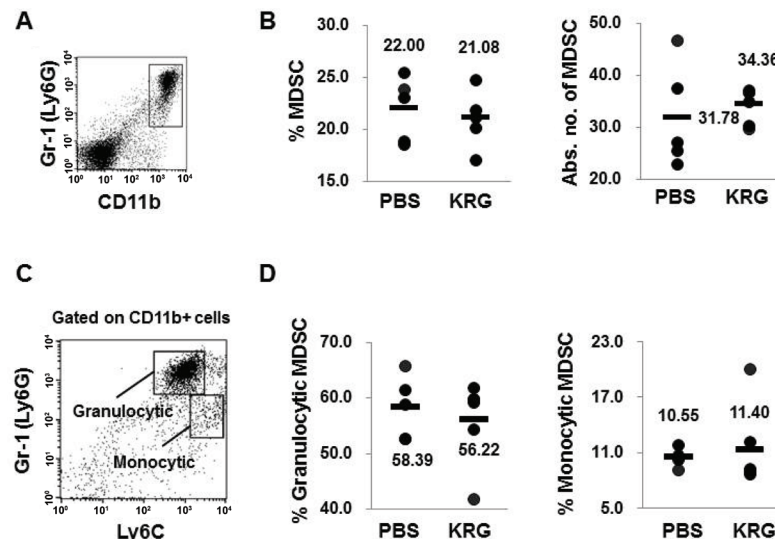


**Fig. 2**. Korean red ginseng (KRG) treatment does not alter the frequency, absolute number, and subset composition of myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice. (A) Flow cytometric strategy used to identify MDSCs. (B) Frequency and absolute number of MDSCs from KRG- or phosphate-buffered saline (PBS)-treated animals. The absolute number of MDSCs was calculated by multiplying the total splenocyte number by the frequency of MDSCs. (C) Flow cytometric analysis of CD11b$^+$Ly6G$^{hi}$Ly6C$^{lo}$ granulocytic and CD11b$^+$Ly6G$^{lo}$Ly6C$^{hi}$ monocytic MDSCs. (D) The frequency of granulocytic and monocytic MDSCs from KRG- or PBS-treated mice.
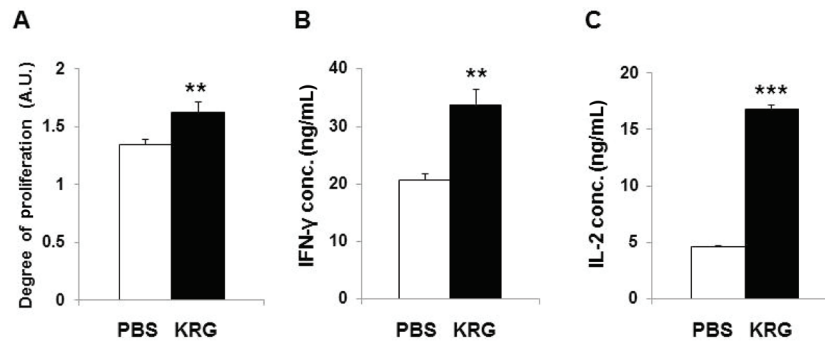
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**Fig. 3.** Inhibition of T cell suppressive effects of myeloid-derived suppressor cells (MDSCs) by Korean red ginseng (KRG) treatment. T cells were stimulated with anti-CD3 Ab and anti-CD28 Ab, co-cultured with MDSCs isolated from KRG- or phosphate-buffered saline (PBS) -treated mice for 4 d, and assessed for their proliferation and cytokine secretion. (A) T cell proliferation. Proliferation is expressed as arbitrary units (A.U.) of absorbance obtained using a CCK assay. IFN-$\gamma$ (B) and IL-2 (C) production measured by enzyme-linked immunosorbent assay. All values represent mean±standard deviation. *$p$<0.05, **$p$<0.01.

### Korean red ginseng inhibits myeloid-derived suppressor cells from suppressing T cell activation

MDSCs have been shown to suppress T cell activation in most patients and experimental animals with tumors. To examine if KRG improves T cell function by inhibiting MDSCs, we measured proliferation and secretion of IL-2 and IFN-$\gamma$ from T cells which were stimulated and co-cultured with MDSCs obtained from KRG or PBS-treated mice. IL-2 is produced mainly by CD4$^+$ T cells functioning as a growth, survival, and differentiation factor for T cells, and IFN-$\gamma$ is produced by CD4$^+$ T cells, CD8$^+$ T cells, and NK cells serving critical functions both in innate and adaptive cell-mediated immunity. Therefore, the two T cell-producing cytokines can be good markers to evaluate T cell activity. We observed that T cell proliferation is enhanced significantly (Fig. 3A), and the release of IL-2 and IFN-$\gamma$ is also greatly increased by KRG treatment (Fig. 3B, C). Our data suggest that although KRG does not block the generation and accumulation of MDSCs, it is able to inhibit the suppressive function of MDSCs and lead to an increase in T cell activation.

### Korean red ginseng impedes the immunosuppressive effects of myeloid-derived suppressor cells

MDSCs use diverse mechanisms to suppress T cell function. They uptake and degrade arginine, an essential amino acid for T cell activation from the microenvironment, using high levels of intracellular arginase. MDSCs, especially monocytic MDSCs, suppress T cell activation via NO-mediated interference of signaling pathways, impede tumor immunity by producing the type 2 cytokine IL-10, and by skewing the immune response toward a tumor-promoting type 2 phenotype [33]. Accordingly, we assessed the mRNA expression of arginase, iNOS, and IL-10, which are the standard markers used to evaluate the suppressive activity of MDSCs. As shown in Fig. 4A and 4B, we observed significant decreases in the levels of iNOS and IL-10 while the expression of arginase was intact in the KRG-treated MDSCs. Furthermore, in support of the decreased expression of iNOS mRNA, MDSCs isolated from KRG-treated mice produced about 5 times less NO compared with MDSCs from the placebo group (2.04 *vs*. 0.39 μM) (Fig. 4C). Taken together, our data indicate that KRG inhibits the T cell suppressive function of MDSCs by downregulating iNOS and IL-10.

## DISCUSSION

Here we provide the first report which evaluates the anti-tumor effects of the medicinal herb KRG by examining the immunosuppressive response of MDSCs. Despite advances in diagnosis, surgical techniques, and new drug development, the prognosis of cancer still remains poor largely due to the resistance of tumor cells to cancer therapies, such as chemotherapy, radiation therapy, and immunotherapy. Many cancer cells acquire diverse resistance mechanisms, and therefore, novel therapeutic approaches are necessary to address the emerging problem of resistance.

One of the strategies cancer cells employ to develop resistance to immunotherapy is to generate cellular suppressors of antitumor immunity. Recent studies have shown that naturally occurring or tumor-driven CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells and immature myeloid or dendritic cells including MDSCs build an immunosuppressive environment in tumor-bearing hosts and are an impediment to immunotherapy [36]. Therefore, to exert effective anti-tumor effects and eliminate tumors,
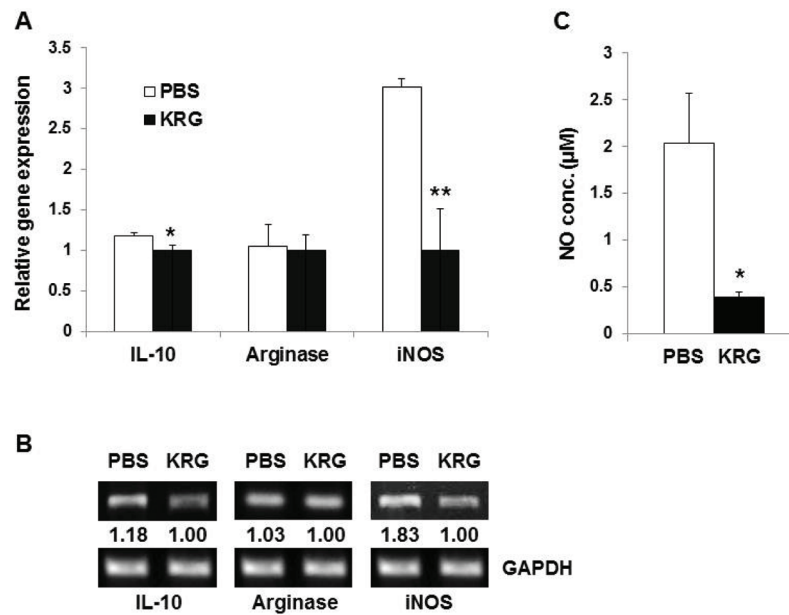
**Fig. 4.** Korean red ginseng (KRG) decreased the expression of IL-10 and inducible nitric oxide synthase (iNOS) in myeloid-derived suppressor cells (MDSCs). MDSCs were isolated from KRG- or phosphate-buffered saline (PBS)-treated mice and stimulated with LPS for 24 h prior to mRNA extraction and collection of culture supernatants. (A,B) The expression of IL-10, arginase, and iNOS mRNA in MDSCs following LPS stimulation. The numbers between gel bands represent quantified and normalized intensities of IL-10, arginase, and iNOS bands which were measured by ImageJ (B). (C) Production of nitric oxide (NO) from MDSCs isolated from KRG- or PBS-treated mice. $*p<0.05$, $**p<0.01$.

such immunosuppressive factors must be overcome.

Many studies have reported that KRG treatment shows anti-tumor activity by directly eradicating tumor cells; additionally, its isolated active ingredients, such as ginsenoside $Rg_3$, $Rp_1$, $Rk_1$, and $Rh_2$, are known to suppress tumor growth through various mechanisms associated with apoptosis, the cell cycle, and ABC transporters [33,37-39]. It is also possible that KRG may overcome tumor cells by stimulating immune cells, but the immunomodulatory function of KRG has been mainly focused on its application as an immune-boosting adjuvant. In light of the lack of in-depth study on the immunostimulatory and anti-tumor functions of KRG, this study delivers very interesting implications.

Although we observed augmented T cell activation in the context of proliferation and cytokine secretion, KRG intraperitoneal administration did not exhibit direct cytotoxicity on tumor cells. However, given that MDSC expansion is accompanied by tumor growth and, conversely, tumor growth is stimulated by the presence of MDSCs, the direct effects of KRG on MDSCs might have been obscured if the tumor size were reduced by KRG. That is, it would not have been clear whether KRG directly affected MDSCs or tumor cells to have anti-tumor effects if a decrease in tumor growth had occurred. However, since we observed improved T cell function

while the sizes of tumor and MDSC were intact, it is more likely that the immunostimulatory effects of KRG results directly from the suppression of MDSCs and is not due to the decrease in MDSC numbers resulting from tumor growth reduction.

$Gr1^+CD11b^+$ MDSCs are heterogeneous and can be further fractionated into morphologically, phenotypically, and functionally distinguishable granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). G-MDSCs mainly exert suppressive functions through ROS and require antigen-specific interactions between MDSCs and T cells, whereas M-MDSCs chiefly use iNOS, arginase, and immunosuppressive cytokines and effectively suppress T cell responses in an antigen-independent manner [40,41]. Studies have reported that M-MDSCs are more potent than G-MDSCs on a per cell basis [9,42]. However, G-MDSCs are mostly present in peripheral lymphoid organs in most tumor models, while M-MDSCs are preferentially tumor-infiltrated [8]. In accordance with previous findings, we observed that many more G-MDSCs are present in the spleen compared with M-MDSCs in our tumor model.

As our co-culture system supported antigen non-specific interactions between MDSCs and $CD4^+$ T cells, we mainly focused on the functional changes in M-MDSCs by KRG treatment and investigated the expression of

iNOS, arginase, and IL-10. Although M-MDSCs are the less prevalent type in the spleen, we were able to see significant declines in the levels of IL-10 and iNOS accompanied by a decrease in NO production. In addition, the extent of M-MDSCs among the whole splenic MDSCs was sufficient to detect KRG-mediated suppression of MDSCs, which led to an increase of T cell activation. We also tried to measure the production of IL-10 and TGF-β by ELISA expecting to see changes in the secretion of immunosuppressive cytokines by KRG, but we were not able to detect them. More sophisticated studies using purified M-MDSCs by sorting would create better results with a more discernible distinction. Furthermore, the use of T cell receptor transgenic mice as a source of tumor antigen-specific CD4$^+$ T cells would be helpful to evaluate the effects of KRG on G-MDSCs.

Taken together, the present study demonstrates that KRG extract enhances T cell proliferation and cytokine secretion by inhibiting the immunosuppressive activity of MDSCs. This study suggests that although KRG alone does not exhibit strong anti-tumor effects, the use of KRG together with conventional chemotherapy or immunotherapy may provide better outcomes to cancer patients through the modulation of MDSC immunosuppressive response.

## ACKNOWLEDGEMENTS

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