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Ginsenosides from Korean red ginseng modulate T cell function via the regulation of NF-AT-mediated IL-2 production

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Abstract Korean red ginseng is a traditional health food frequently used to prevent or treat various diseases worldwide. In this study, we evaluated the immunomodulatory activities of eleven compounds (**1–11**) isolated from Korean red ginseng, focusing on T cell function. First, the effects of the eleven compounds were studied on the regulation of IL-2, a potent T cell growth factor. Compounds **5**, **7**, and **9** significantly increased IL-2 secretion in phorbol 12-myristate 13-acetate (PMA)/ionomycin (Io)-induced EL-4 T cells. Next, we examined the effects of compounds **5**, **7**, and **9** on the regulation of transcription factors related to IL-2 production in T cells. Compound **9** significantly increased the PMA/Io-induced promoter activity of nuclear factor of activated T cells (NF-AT) in EL-4 T cells, but did not have any significant effects on the promoters of NF-κB. These results suggest that compound **9** activates T cell function via the regulation of NF-AT-mediated IL-2 production.

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Introduction

Korean red ginseng (original scientific name as *Panax ginseng*, Araliaceae) is the best-known processed ginseng product in Korea. For thousands of years, it has been used as an herbal medicine and health food in countries such as South Korea, China, and Japan (Jia et al., 2009). Dammarane type triterpene saponins (ginsenosides) are known as the major effective component in Korean red ginseng. Ginsenosides have many biological activities including anti-cancer, anti-inflammation, anti-tumor, anti-diabetes, and immunomodulatory effects (Attele et al., 1999; He et al., 2017; Jiang et al., 2017). Recently, more than 80 ginsenosides have been isolated from various parts of *P. ginseng*. Ginsenosides Rg1 and Rd were reported to have immunomodulatory effects on T cells (Lee and Han, 2006; Yang et al., 2007). Although many ginsenosides have been isolated and their biological activities described, it is still important to find new ginsenosides from Korean red ginseng and to clarify their various biological activities and action mechanisms.

Communication between the innate and adaptive immune systems is crucial for successful immunity to pathogens. The adaptive immune system is strongly influenced by innate effector cells, and adaptive responses to pathogen infection require synergistic collaboration with the innate immune system. T cells are critical for the generation of effective immune reactions to a pathogen. In addition, T helper cells regulate cytokine secretion and engage in aspects of cell-to-cell signaling to promote immune responses (Kara et al., 2014). Interleukin-2 (IL-2)

is the main stimulator of T cell differentiation and growth (Read et al., 2016). IL-2 drives the clonal expansion of activated T cells and prompts cell differentiation to acquire effector functions (Smith, 1992). Consequently, the specific production of IL-2 is an important step in T cell-dependent immune responses. The production of IL-2 is mainly regulated at the transcriptional level through multiple transcription factors. The nuclear factor of activated T cells (NF-AT) associated with activator protein-1 (AP-1) has been reported to bind several motifs within the IL-2 promoter. The binding of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the IL-2 promoter activates a canonical site for NF-κB and transcription of the CD28 response element (Macian, 2005). Cyclosporine A and tacrolimus inhibit IL-2 production and represent the mainstay of immunosuppressive therapy in transplantation. Their mechanism of action is the inactivation of the Ca^{*}/-calmodulin-dependent serine-threonine phosphatase, calcineurin, leading to the inactivation of NF-AT, a transcription factor that is required for the expression of IL-2, interferon-γ (IFN-γ), and granulocyte–macrophage colony- stimulating factor (GM-CSF) genes (Liu, 2009).

Previously, we isolated eleven compounds, including two new ginsenosides from Korean red ginseng and evaluated their anti-inflammatory activities (Vinh et al., 2017). These results showed that compound **1** (3-oxo-20*R*-ginsenoside Rh1) significantly reduced the level of proinflammatory cytokines IL-6 and TNF-α secretion and inhibited the expression of COX-2 and iNOS in LPS-activated RAW264.7 cells.

In this study, we evaluated the immunomodulatory activities of the eleven compounds (**1–11**), focusing on T cell functions. Compounds **5**, **7**, and **9** significantly increased IL-2 secretion in phorbol 12-myristate 13-acetate (PMA)/ionomycin (Io)-induced EL-4 T cells, and we tested the effects of compounds **5**, **7**, and **9** on the regulation of transcription factors related to IL-2 production in T cells.

Materials and methods

Cell cultures

EL-4 T cells purchased from the American Type Culture Collection (Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene; Daegu, Korea) supplemented with 1% penicillin/streptomycin (Thermofisher Scientific; Waltham, MA, USA)and 10% FBS (Thermofisher Scientific; Waltham, MA, USA) under an atmosphere of 5% CO_2 in a humidified 37 °C incubator.

Cell proliferation assay

EL-4 T cells (1×10^4 cells/well) were treated with each compound (5 and 10 μM) for 48 h as previously described (Park et al., 2017a). Viability of EL-4 T cell was assayed by using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (CCK-8, Dojindo; Rockville, MD, USA). Absorbance was read with an ELx808 ELISA microplate reader (BioTek Instruments Inc.; Winooski, VT, USA).

Enzyme-linked immunosorbent assay (ELISA)

EL-4 T cells (1×10^5 cells/well) were pretreated with each compound for 4 h followed by the addition of phorbol 12-myristate 13-acetate (PMA, 0.5 ng/mL)(Sigma-Aldrich Chemical Co; St. Louis, MO, USA)/ionomycin (Io, 5 nM)(Sigma-Aldrich Chemical Co; St. Louis, MO, USA) for 48 h as previously described (Park et al., 2017a). The IL-2 cytokines released in the cell culture media were measured using sandwich ELISA kits following the manufacturer's experimental protocols (R&D Systems; Minneapolis, MN, USA). The absorbance was measured at 450 nm using an ELISA microplate reader within 30 min.

DNA transfection and luciferase reporter assays

NF-AT or NF-κB promoter driven plasmids pNF-AT-Luc and pNF-κB-Luc, respectively, were purchased from Promega (Madison, WI, USA). EL-4 T cells were seeded in a 6-well plate (5×10^5 cells/well) and transfected with the indicated firefly luciferase reporter DNAs (3 μg) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as previously described (Park et al., 2017a). After a 48 h incubation, the cells were treated with each compound for 4 h, followed by treatment with PMA (0.5 ng/mL)/Io (5 nM) for 24 h. Luciferase activity in the cell lysates was measured with the luciferase reporter system (Promega, Madison, WI, USA).

Results and discussion

Phytochemical analysis

The extract of Korean red ginseng was subjected to various separation procedures and 11 compounds were isolated (**1-11**). Their structures were identified as 6-*O*-*β*-D-glucopyranosyl-20*R*-protopanaxadiol-3-one (**1**), 20*R*-ginsenoside Rh1 6′-acetate (**2**), 20*S*-ginsenoside Rh1 6′-acetate (**3**), ginsenoside Rk3 (**4**), ginsenoside RT5 (**5**), 20*R*-ginsenoside Rh1 (**6**), 20*S*-ginsenoside Rh2 (**7**), 20*R*-ginsenoside Rg3 (**8**), oleanolic acid *β*-D-glucopyranosyl ester (**9**),

ginsenoside Rh4 (**10**), and 9,12,13-trihydroxy-10*E*-octadecenoic acid (**11**) based on spectral and chemical evidence, consistent with previously published paper (Vinh et al., 2017) (Fig. 1).

Biological activities

To study the immunomodulatory activity, we evaluated the effectiveness of eleven compounds (**1–11**) on IL-2 cytokine production in PMA/Io-induced EL-4 T lymphocyte cells (ATCC, Manassas, VA, USA). IL-2 plays an essential role in early T lymphocyte clonal differentiation and expansion (Gaffen and Liu, 2004; Rochman et al., 2009).

The effect of the eleven compounds isolated from Korean red ginseng was evaluated on the production of a potent T cell growth factor IL-2. Among tested eleven ginsenosides, compounds **5**, **7**, and **9** significantly increased the production of IL-2 cytokine from PMA/Io-activated EL-4 T cells in a dose-dependent manner [Fig. 2(A), (B)]. Compound **9** at all tested concentrations of 2.5, 5.0, and 10 μM significantly increased IL-2 production from EL-4T cells pre-treated with PMA/Io.

Next, we investigated the possibility that IL-2 increase induced by compounds **5**, **7**, and **9** was related to regulation of transcriptional factors, NF-AT and NF-κB. NF-AT is an essential transcriptional factor for IL-2 expression by activated T cells (Macian, 2005; Maloy and Powrie, 2001). NF-κB also binds to the IL-2 promoter. EL-4 T cells were transfected with each firefly luciferase reporter NF-AT and NF-κB promoters, and then treated with the three compounds for 2 h and PMA/Io for 24 h. Compounds **5** and **7** did not show any effects on NF-AT or NF-κB promoter activity (Fig. 3). Compound **9** (oleanolic acid *β*-D-glucopyranosyl ester) significantly increased NF-AT promoter activity at a concentration of 10 μM in PMA/Io-activated EL-4 T cells [Fig. 3(A)], while no difference was observed in the NF-κB promoter activity [Fig. 3(B)]. These results suggest that compound **9** exerts its immunomodulatory activity by upregulating T cell growth factor IL-2 production via the regulation of NF-AT. Activated $CD4^+$ and

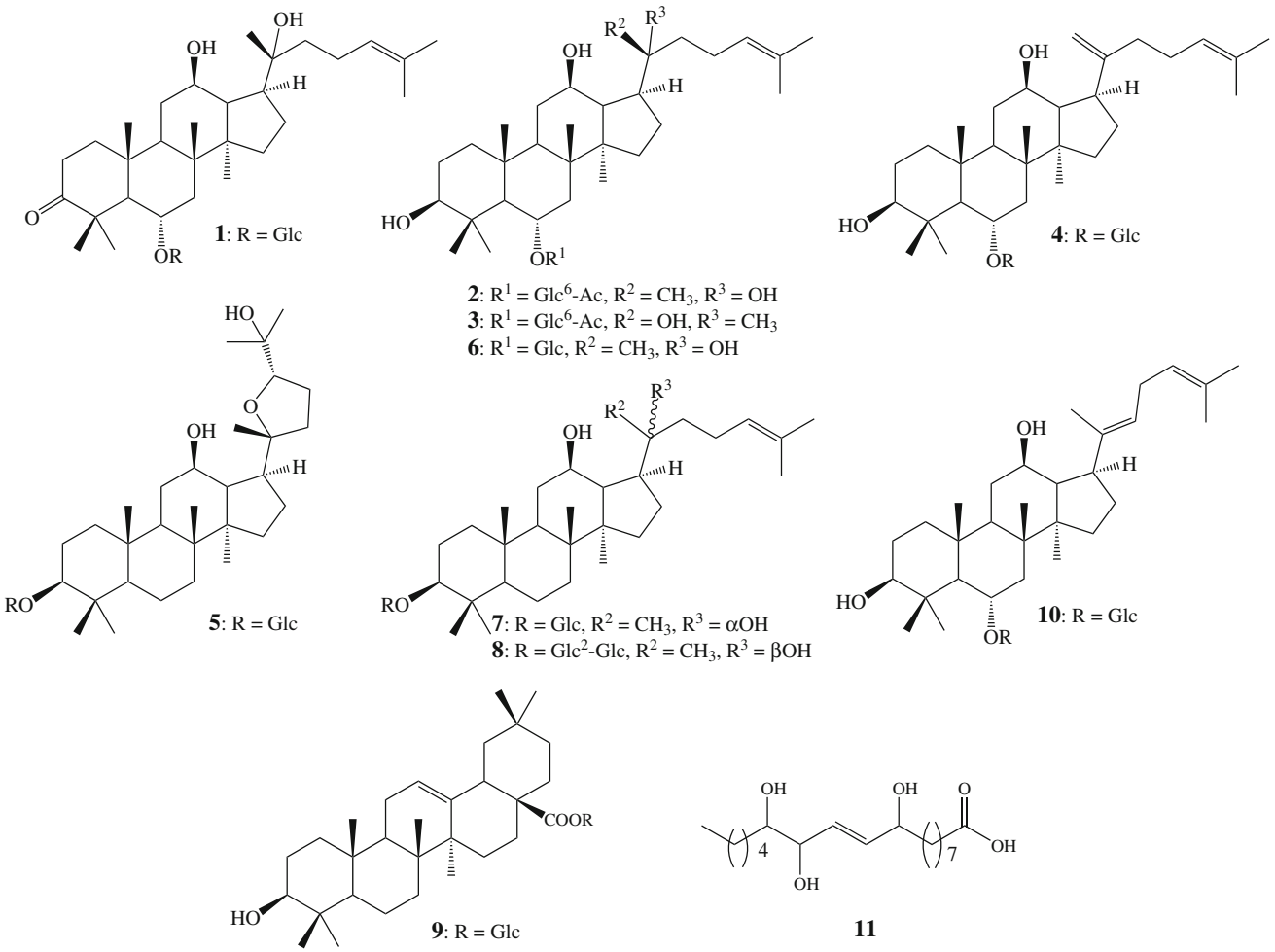

Fig. 1 Structure of ginsenosides from Korean red ginseng

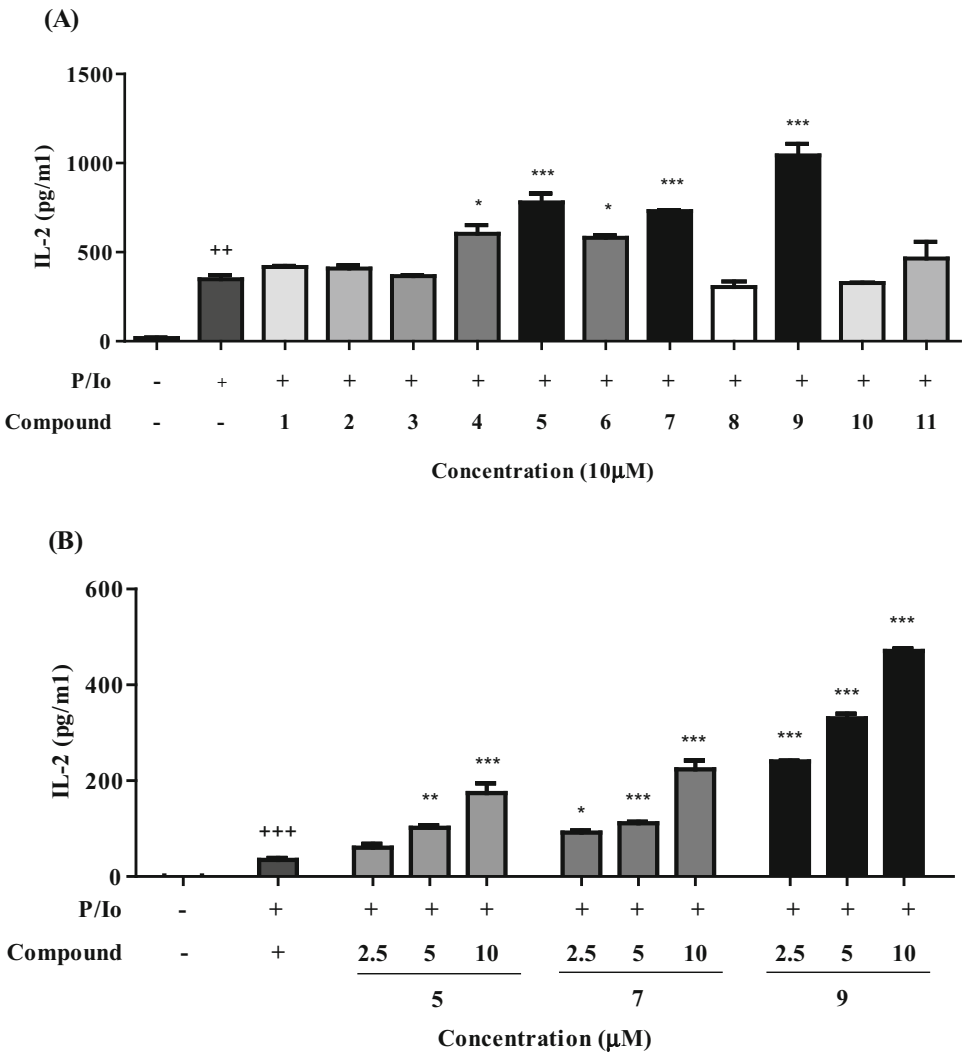

Fig. 2 Effect of compounds on IL-2 production in PMA/Io-induced EL-4 T cells

$CD8^+$ T cells are usually regulated by multiple transcription factors including NF-AT, NF-kB, AP-1, and CREB/AFT (Kim et al., 2006). These results suggest that the compounds **5** and **7** could induce IL-2 production by transcription factors other than NF-AT and NF-kB. Further study would elucidate the mechanism by which the compounds **5** and **7** increase IL-2 production from EL-4 T cells.

In this study, we found for the first time that compounds **5** and **7** had the immunomodulatory activity increasing the production of IL-2 cytokine, but not related with NF-AT and NF-κB promoter activity. Compound **5** (ginsenoside RT5) was reported to have cytotoxic effects (Atopkina et al., 1999). Recently, compound **7** (20*S*-ginsenoside Rh2) was identified as a major metabolite of ginseng that had antitumor activity and the potential to prevent cancer (Chen et al., 2017; Shi et al., 2016). Moreover, it has been shown to induce the apoptosis and differentiation of acute myeloid leukemia cells (Wang et al., 2017).

It also indicates that oleanane type triterpenoids have greater immunomodulatory activity than does the dammarane type.

We also tested single compounds (**5**, **7**, and **9**) on the viability of the T cells by assessing cell metabolic activity (Park et al., 2017b). Compounds **5**, **7**, and **9** did not show any cytotoxic effect on EL-4 T cells at concentrations of 5 and 10 μM (Fig. 4). In this study, the compounds **5**, **7**, and **9** increased IL-2 productions from EL-4 T cells (Fig. 2), but did not show any activity on the cell proliferation (Fig. 4). IL-2 cytokine is the main regulator of survival, proliferation, and differentiation of activated T cells and NK cells. IL-2 shows dual functions in the immune response. IL-2 not only induces effector lymphocyte responses but also regulates or inhibits the responses at the same time by driving the expansion and suppressive function of regulatory T cells (Sim et al., 2014; Sim and Radvanyi, 2014; Williams et al., 2006). IL-2 was reported

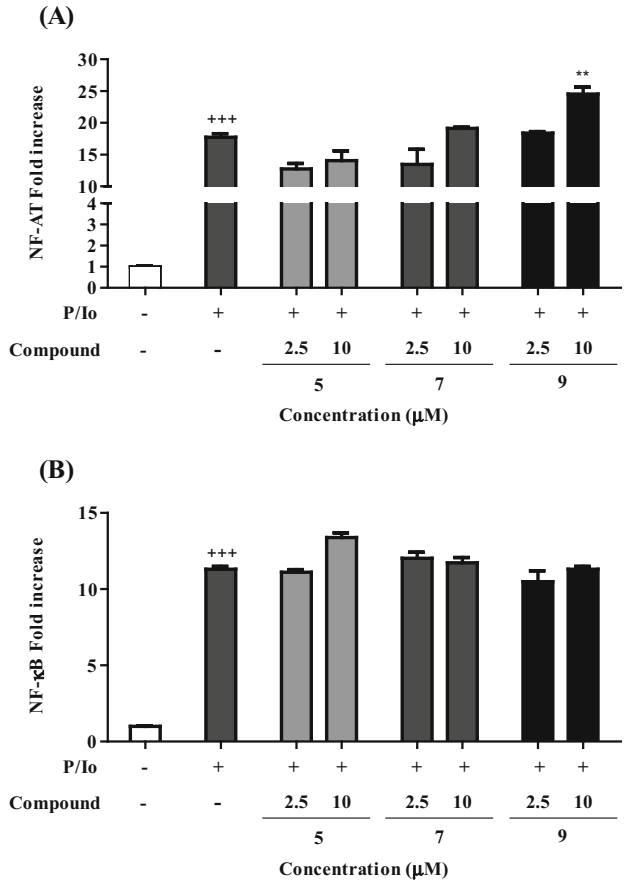

Fig. 3 Effect of compounds **5**, **7**, and **9** on NF-AT and NF-κB promoter activities in PMA/Io-activated EL-4 T cells

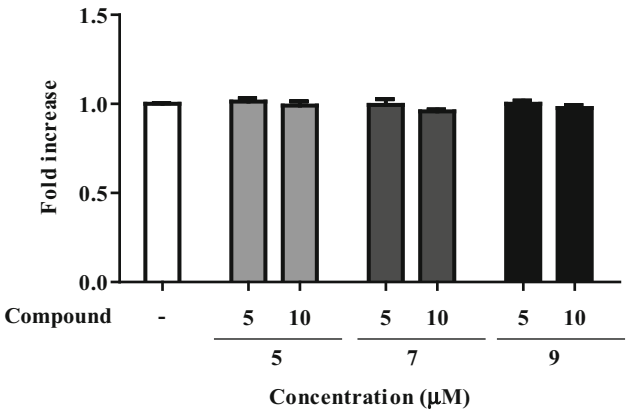

Fig. 4 Effects of compounds **5**, **7**, and **9** on the viability of EL-4 T cells

to have a narrow therapeutic window, and the level of dosing should be determined by further study.

Recently, studies of oleanane type triterpenoid saponins were investigated with good effects of immunomodulatory, hemolytic, and cytotoxic properties while studies about ginsenosides (dammarane type saponins) have limited in immunomodulatory activities (Sarikahya et al., 2018).

This is the first study reporting of the immunomodulatory activity of compounds **5, 7**, and **9** obtained from a Korean red ginseng. In the view point of structure activity relationship, oleanane type triterpenoids have more potent immunomodulatory activity than does the dammarane type.

Immune responses are important in the prevention and treatment of infectious diseases and cancer. Thousands of studies have described the diverse roles of compounds isolated from *P. ginseng* in physiological processes such as cancer, neurodegenerative disorders, insulin resistance, and hypertension. *P. ginseng* facilitates homeostasis of the immune system and to enhance resistance to illness or microbial attacks via regulation of the immune system (Kang and Min, 2012).

The present study suggests that compounds **5**, **7**, and **9** (ginsenosides from Korean red ginseng) show immune enhancing activity through the modulation of T cell function caused by the increased IL-2 production. Considering the important roles of T cells in immune responses, these results suggest that compounds **5**, **7**, and **9** showing T cell-mediated immune potentiating activity have potential effects for a cure of cancer immune therapy.

Given the results for compounds **5**, **7**, and **9**, these compounds are candidates for further investigation to elucidate their molecular mechanisms of action on specific immunomodulatory targets.

EL-4 T cells were pretreated with each compounds for 4 h and then incubated with PMA (0.5 ng/mL)/Io (5 nM) for 48 h. An ELISA was used to measure IL-2 production in cell culture medium. Compounds **5**, **7**, and **9** increased IL-2 cytokine production in EL-4 T cells activated with PMA/Io. All statistical calculations were performed using GraphPad Prism ver. 5.01 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance followed by the Bonferroni post hoc test was used for multigroup comparisons. ($+P < 0.01$ and $+++P < 0.001$ in comparison with the untreated group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ correlated with the PMA/Io-treated group.)

EL-4 T cells were transfected with the reporter plasmids pNF-AT-Luc (A) or pNF-κB-Luc (B). The EL-4 T cells were pretreated with each compounds for 4 h and then incubated with PMA (0.5 ng/mL)/Io (5 nM) for 24 h. Luciferase activity in cell lysates were measured with the luciferase reporter system. All statistical calculations were performed using GraphPad Prism ver. 5.01 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance followed by the Bonferroni post hoc test was used for multigroup comparisons. ($+P < 0.01$ and $+++P < 0.001$ in comparison with the untreated group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ correlated with the PMA/Io-treated group.)

EL-4 T cells were exposed to single compounds **5**, **7**, and **9** for 48 h and the cell cytotoxicity was assayed by

CCK-8. Compound **5**, **7**, and **9** did not show cytotoxic effect on EL-4 T cells at concentrations of 5 and 10 μM. $*P < 0.05$ and $***P < 0.001$ compared to the untreated group.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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