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Immune Enhancing Effects of *Echinacea purpurea* Root Extract by Reducing Regulatory T Cell Number and Function

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Echinacea purpurea preparations (EPs) have been traditionally used for the treatment of various infections and also for wound healing. Accumulating evidence suggests their immunostimulatory effects. Regulatory T cells (Tregs) are known to play a key role in immune regulation *in vivo*. However, there have been no reports so far on the effects of EP on the frequency or function of Tregs *in vivo*. Therefore, in the present study, we investigated the quantitative and functional changes in Tregs by *in vivo* administration with EP. The frequencies of $CD4^+FoxP3^+$ and $CD4^+CD25^+$ Tregs in the spleens of BALB/c mice administered with EP for 3 weeks were investigated by flow cytometry. The suppressive function of $CD4^+CD25^+$ Tregs in association with the proliferative activity of $CD4^+CD25^-$ effector T cells (Teffs) and the feeder function of $CD4^-$ antigen-presenting cells (APCs) were analyzed by carboxyfluorescein succinimidyl ester-dilution assay. The results showed a lowered frequency of $CD4^+FoxP3^+$ and $CD4^+CD25^+$ Tregs and attenuated suppressive function of $CD4^+CD25^+$ Tregs, while the feeder function of APCs was enhanced in the EP-administered mice. On the other hand, the proliferative activity of Teffs was not significantly different in the EP-administered mice. The results suggest that decreased number and function of Tregs, in association with the enhanced feeder function of APCs, may contribute to the enhancement of immune function by EP.

Keywords: *Echinacea purpurea*, Immunomodulation, Regulatory T cell, FoxP3.

Echinacea purpurea has long been used as a traditional herbal medicine for the treatment of common cold in North America [1a]. Recent studies showed that *E. purpurea* preparations (EPs) significantly reduced the duration and/or severity of upper respiratory infections (URIs) [1b,c,2]. EPs were also reported to be effective in the clinical improvement of many infectious diseases including skin disorders [3a,b]. Experimental studies showed that extracts of *E. purpurea* have antimicrobial activity [3-6].

The immune-enhancing effects of *Echinacea* are well-documented and the underlying mechanisms have been widely investigated [4b,6,7]. The immunological mechanisms accumulated so far include enhanced phagocytic activity and macrophage activation as well as enhanced NK cell activity, suggesting the activation of an innate immune system [8a-8c]. Additionally, mitogen-stimulated lymphocyte proliferation and specific antibody production were also enhanced, suggesting that *Echinacea* may also activate the adaptive immune system [8d,9a,b]. Other noticeable findings reported increased secretion of several cytokines, including TNF-α, IL-1, and IL-6 from monocytes and/or dendritic cells [7a,10a].

Regulatory T cells (Tregs) are known to play a key role in the maintenance of immune homeostasis *in vivo* [10b]. Although Tregs were originally identified in the $CD4^+CD25^+$ fraction for their critical role in preventing the development of autoimmune diseases, they are also important in the regulation of most of the immune responses, including infection, allergic reactions, graft rejection and anti-tumor immunity [10c]. Tregs exert their regulatory effects by suppressing the proliferation and function of immune effector cells including $CD4^+$ helper T cells [10d], $CD8^+$ cytotoxic lymphocytes [10e], B cells [10f] and NK cells [10g]. Thus, Tregs regulate both innate and adaptive immune systems *in vivo*. However, there are no reports on how EPs influence either the frequency or function of Tregs *in vivo*. Among the many molecules that may distinguish Tregs from other lymphocyte subsets, FoxP3 is the most specific marker identified so far [10h]. Deficiency of FoxP3 results in lymphoproliferative and autoimmune disorders due to agenesis of Tregs [11,12]. Forced expression of FoxP3 in effector T cells conferred suppressive function and similar phenotype as Tregs [13a]. Therefore, it can be argued that FoxP3 is a master gene regulating the development and function of Tregs. In the present study, in order to test the effect of EP on the number and function of Tregs *in vivo*, we investigated the number of $CD4^+FoxP3^+$ and $CD4^+CD25^+$ cells, as well as the suppressive function of $CD4^+CD25^+$ cells in spleens from BALB/c mice orally administered with EP for 3 weeks. The results indicated that oral administration of EP decreased the number of $CD4^+FoxP3^+$ and $CD4^+CD25^+$ cells, as well as suppressive function of $CD4^+CD25^+$ cells. Flow cytometric analysis showed that the percentage and absolute counts of $CD4^+CD25^+$ and $CD4^+FoxP3^+$ fractions in splenocytes obtained from mice administered with EP were found to be significantly lower than those from control mice (Figure 1), suggesting that EP administration reduced the production and/or survival of Tregs.

Carboxyfluorescein succinimidyl ester-dilution assay showed that the proliferative response of $CD4^+CD25^-$ Teffs from the EP-administered mice was comparable with those from control mice (Figure 2A, B, C). By contrast, the proliferative responses of Teffs in the presence of $CD4^-$ APCs from the EP-administered mice were significantly stronger than those in the presence of $CD4^-$ APCs from control mice, irrespective of the source of Teffs (Figure 2A, D, E). A stronger proliferative response of Teffs in the presence of APCs from the EP-administered mice was also observed in co-culture with $CD4^+CD25^+$ Tregs (Figure 3). Taken together, it was suggested that $CD4^-$ APCs from the EP-administered mice provided better supportive signals necessary for the proliferation of $CD4^+CD25^-$ Teffs than those from control mice.

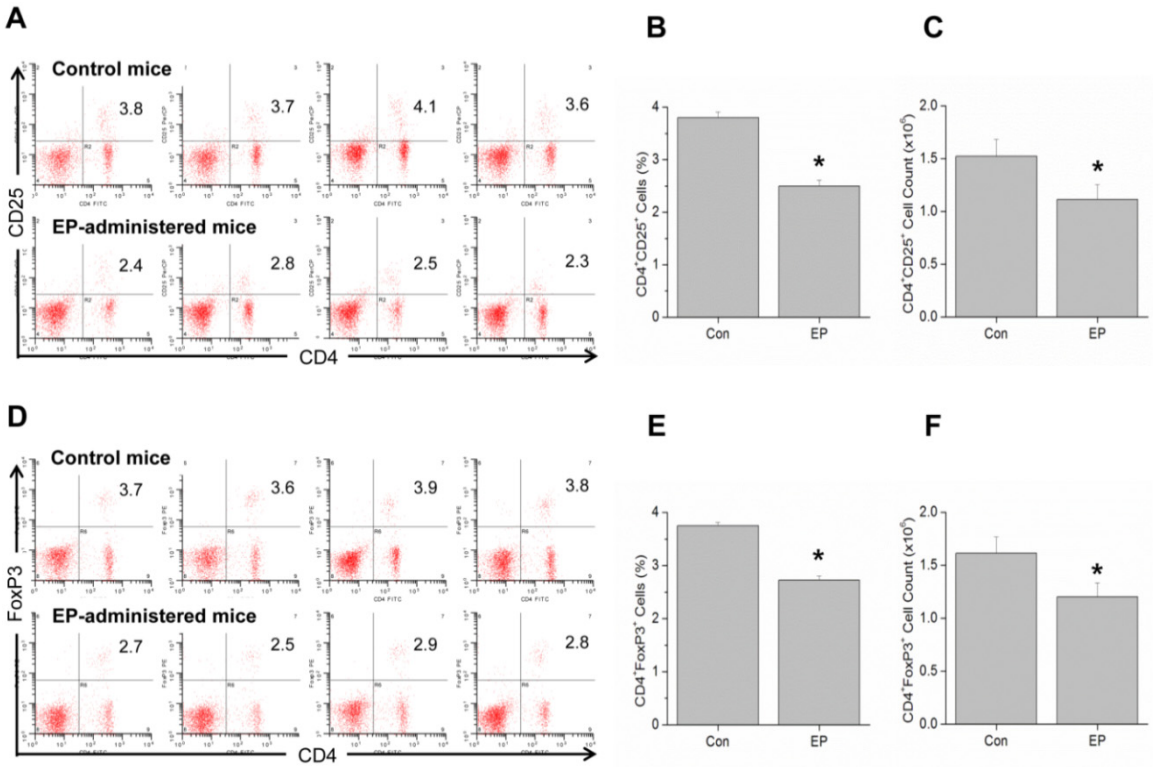

Figure 1: Administration of *E. purpurea* preparation (EP) decreased the proportions and absolute counts of $CD4^+CD25^+$ and $CD4^+FoxP3^+$ fractions. The proportion of the $CD4^+CD25^+$ fraction in the splenocytes from EP-administered mice (lower row) was significantly lower than those of control mice (upper row) (A, B). The absolute count of $CD4^+CD25^+$ fraction in the splenocytes from EP-administered mice was also significantly lower than those of control mice (C). The proportion of $CD4^+FoxP3^+$ fraction in the spleens of the EP-administered mice (lower row) was significantly lower than those of the control mice (upper row) (D, E). The absolute count of the $CD4^+FoxP3^+$ fraction in splenocytes from EP-administered mice was also significantly lower than those of control mice (F). Data are mean ± SD (n = 4). A representative series of FACS plots of 3 separate experiments. Comparison of data was made by using independent Student's *t* test. $^*P < 0.05$.

For the functional study of Tregs, we investigated the suppressive function of only $CD4^+CD25^+$ cells, since it is still technically difficult to isolate live $FoxP3^+$ cells as they cannot be isolated without intranuclear staining. In the spleens from BALB/c mice, the $CD4^+CD25^+$ fraction substantially overlap with $CD4^+FoxP3^+$ fraction; 86.7 + 5.1% (n=20) of $CD4^+CD25^+$ cells were FoxP3 (data not shown) [13b]. Therefore, as Tregs were originally identified in the $CD4^+CD25^+$ fraction, the $CD4^+CD25^+$ fraction reflects the functional characteristics of Tregs quite well and is still popularly used for functional study of Tregs [10b]. The proliferative responses of $CD4^+CD25^-$ Teffs in the co-culture with $CD4^+CD25^+$ Tregs from the EP-administered mice were significantly stronger than with those from control mice, suggesting that the suppressive function of Tregs from the EP-administered mice was weaker than those from control mice (Figure 3A – D). The extent of suppression calculated by comparing the proliferation of Teffs in the presence and absence of Tregs (% Suppression) was significantly different between the Tregs from the EP-administered mice and control mice (Figure 3E, F). The mechanism of decreased number and function of Tregs is beyond the scope of this study. A plausible explanation could be the increased level of IL-6 consistently reported in the EP-administered mice may be related with the low frequency of Tregs [7,10a]. IL-6 and/or IL-21 in association with TGF-β induces the differentiation of naïve T cells into Th17 cells, while inhibiting that into Tregs [13c]. IL-6 and/or IL-21 also inhibit the suppressive function of Tregs [13b,d].

A combination of each type of cells that take part in the immune response isolated from the same mice may reflect their *in vivo* immune. In order to simulate the *in vivo* immune responsiveness of the EP-administered mice, the proliferative responsiveness of Teffs in the cognate combinations of APCs (and Tregs) from the EP-administered mice were compared with those from control mice (Figure 4A and B, respectively). The results show stronger proliferative responsiveness of Teffs in the cognate cell combinations of the EP-administered mice, suggesting that EP administration enhanced the immune responsiveness.

Experimental

Preparation of ethanol extract of Echinacea purpurea: An ethanol extract of *E. purpurea* root (available as extract powder) was obtained from Tasman Extracts Ltd. (Nelson, New Zealand). Chicoric acid, chlorogenic acid, cynarin, echinacoside, and dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide were quantified by HPLC. The concentrations of chicoric acid and dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide contained in the ethanol extract from *E. purpurea* root were 8,665.2 ± 33.1 and 218.5 ± 11.7 μg/g, respectively [14]. However, the other compounds, including chlorogenic acid, cynarin, and echinacoside were not detected.

Animals: Male BALB/c mice (23 - 27 g; 6 - 8 weeks of age) were obtained from Daehan Biolink (Eumsung, Korea) and maintained on a 12 h light-dark cycle at a constant temperature of 24 ± 3°C in specific pathogen free conditions. This study was performed according to the Korean Food and Drug Administration guidelines and approved Institutional Animal Care and Use Committee (Permit Number: 10-0133). All surgeries were performed under isoflurane anesthesia and every effort was made to minimize animal suffering. The mice were administered orally with either the *E. purpurea* (300 mg/kg) or DMSO control diluted in PBS every day for 3 weeks.

Quantitative analysis of Tregs by flow cytometry: Spleen cells were prepared from the mice for flow cytometry by squeezing on a cell strainer (70 μm, BD Biosciences, San Jose, CA) and lysing the erythrocytes using ACK lysis buffer. After blocking the Fc receptors using anti-mouse CD16/CD32 (2.4G2) for 15 min at 4°C,

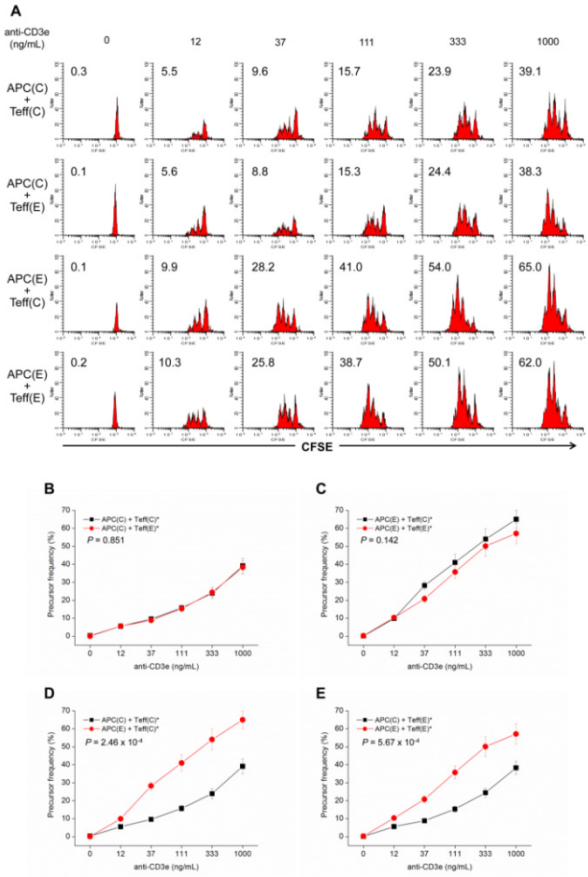

Figure 2: Administration of *E. purpurea* preparation (EP) enhanced the feeder function of $CD4^-$ antigen-presenting cells (APCs). The proliferative response of $CD4^+CD25^-$ Teffs from the EP-administered mice (red) were comparable with those from the control mice (black) (A and B). By contrast, the proliferative responses of $CD4^+CD25^-$ Teffs in the presence of $CD4^-$ APCs from the EP-administered mice (red) were significantly stronger than those in the presence of CD4- APCs from control mice, irrespective of the source of Teffs (black) (C and D), suggesting that $CD4^-$ APCs from the EP-administered mice provided better supportive signals necessary for the proliferation of $CD4^+CD25^-$ Teffs than those from the control mice. Statistical analyses of the data shown in Figure S1. Data are mean ± SD of 3 separate experiments. Comparison of data was made by using one way ANOVA. $*P < 0.05$.

cells were stained for surface antigens with anti-CD4 (H129.19) and anti-CD25 (PC61) for 30 min at 4°C. For intranuclear staining for FoxP3, cells were fixed and permeabilized using a mouse regulatory T cell staining kit (eBiosciences, San Diego, CA) and were stained with FJK-16s-PE-Cy5. Each sample was acquired with a FACSCalibur (BD Biosciences) and was analyzed by using Winlist software (Verity, Topsham, ME).

Preparation of cells: The $CD4^+CD25^+$ fraction was separated from the splenocytes of either the control mice or the EP-administered mice for Tregs by immunomagnetic selection using a regulatory T cell isolation kit from Miltenyi Biotech (Auburn, CA). Purity of $CD4^+CD25^+$ cells, checked by flow cytometry, was from 90% to 95%. The $CD4^+CD25^-$ fraction was separated for the effector T cells (Teffs) and the $CD4^-$ fraction was isolated and used for antigen-presenting cells (APCs) after treatment with mitomycin C. Parts of the $CD4^+CD25^-$ Teffs were labeled with carboxyfluorescein diacetate succinimidyl ester, as described elsewhere [15].

In vitro *proliferative assay:* For the proliferative assay, 10^4 CFSE-labeled Teffs were stimulated with various concentrations of anti-CD3e (e-Biosciences) in the presence of 10^5 APCs. In order to assess the suppressive function of Tregs, 5 x 10^3 unlabeled Tregs was added to the co-culture of 10^4 CFSE-labeled Teffs and 10^5 APCs. The cells were cultured in DMEM supplemented with 10% FCS (Hyclone, Logan, UT) in round-bottomed 96-well plates. On the 3rd day of culture, the cells were harvested for staining with

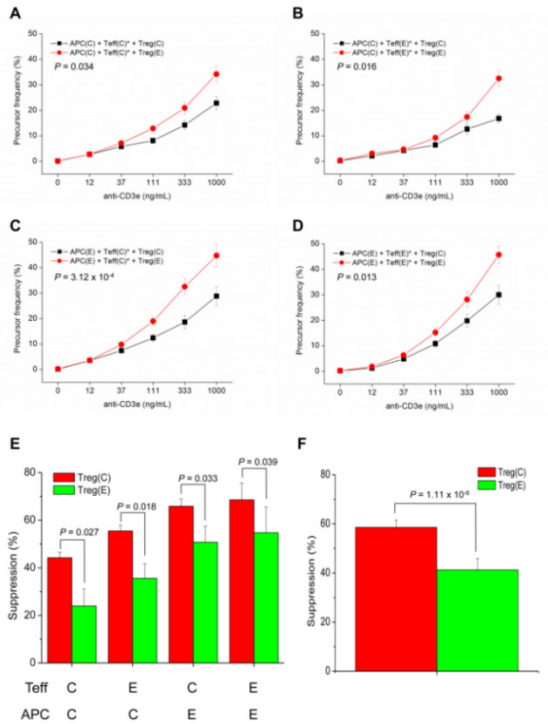

Figure 3: Administration of *E. purpurea* preparation (EP) attenuated the suppressive function of $CD4^+CD25^+$ Tregs. The proliferative responses of $CD4^+CD25^-$ Teffs in the co-culture with $CD4^+CD25^+$ Tregs from the EP-administered mice (red) were significantly stronger than with those from control mice (black) (A – D). Suppression (%) calculated by comparing the proliferation of Teffs in the absence and presence of Tregs are also significantly different between the Tregs from control mice (red) and those from the EP-administered mice (green), irrespective of the source of Teffs and APCs in the co-culture (E). The average suppression (%) by Tregs from the EP-administered mice (green) was approximately 31.2 ± 2.7% (n = 48) less than that from the control mice (red) (F). Statistical analyses of the data shown in Figure S2. Data are mean ± SD of 3 separate experiments. Comparison of data was made by using either one way ANOVA (A – D) or independent Student's *t* test (E and F). $*P < 0.05$.

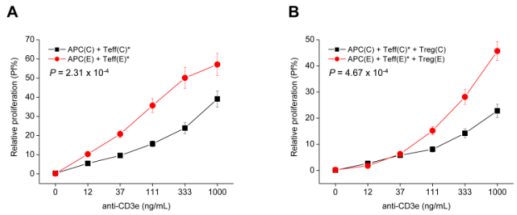

Figure 4: Administration of *E. purpurea* preparation (EP) enhanced immune responsiveness. The proliferative responsiveness of Teffs from the EP-administered mice in the presence of cognate APCs (and Tregs, red) from the same mice was stronger than that from control mice (black). APCs + Teffs combination (A); APCs + Teffs + Tregs combination (B). Data are mean ± SD of 3 separate experiments. Comparison of data was made by using one way ANOVA. $*P < 0.05$.

anti-CD25-PE (BD Biosciences and anti-CD4-PerCP (BD Biosciences). Whole cells were acquired for analysis by using Winlist software (Verity, Topsham, ME). For the precise analysis of the proliferative response of the Teffs, precursor frequency (Pf) was estimated for the cells exclusively gated for $CFSE^+CD4^+$ live cells according to the scattering characteristics using the proliferation wizard of Modifit software (Verity), as described before [13b].

Comparison of immune function before and after the administration of EP in mice: We assessed the immune responsiveness of the EP-administered mice by comparing the functional activities of Teffs, APCs and Tregs with those of the control mice treated with DMSO. Comparisons were made from the results of criss-cross combination of the Teffs, APCs and Tregs from the control and the EP-administered mice (total 12 combinations; 4 combination of the Teffs and APCs; and 8 combinations of the Teffs, APCs and Tregs). For the functional assessment of the Teffs, the Pf values of the Teffs from the control and the EP-administered mice, in the presence of the same APCs,

i.e. from the control or the EP-administered mice, were compared. Similarly, for the functional assessment of APCs, the Pf values of the same Teffs, i.e. from the control or the EP-administered mice, in the presence of APCs from the control and the EP-administered mice were compared. For the functional assessment of Tregs, the Pf values of the Teffs were compared between those in the absence and presence of Tregs, to give rise to the suppressive activity of Tregs (% Suppression). In order to assess the overall immune function of the EP-administered mice, we also compared the Pf values of the Teffs in the presence of cognate APCs (and Tregs), i.e. between the combination of Teffs and APCs (and Tregs) from the control mice and those from the EP-administered mice.

Statistical analysis: Data are expressed as mean ± SD of 3 separate experiments. Comparison of data between two groups was made by using an independent Student's *t*-test, while data from more than two groups were analyzed by one way ANOVA. *P* values less than 0.05 were considered statistically significant.

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