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Alpha-phellandrene Promotes Immune Responses in Normal Mice Through Enhancing Macrophage Phagocytosis and Natural Killer Cell Activities

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Abstract. *α-Phellandrene, a natural compound from natural plants, has been used in the food and perfume industry. We investigated the effects of α-phellandrene on the immune responses on normal murine cells in vivo. Normal BALB/c mice were treated orally with or without α-phellandrene at 0, 1, 5 and 25 mg/kg and olive oil as a positive control for two weeks. Results indicated that α-phellandrene did not change the weight of animals when compared to olive oil (vehicle for α-phellandrene)-treated groups. After flow cytometric assay of blood samples it was shown that α-phellandrene increased the percentage of CD3 (T-cell marker), CD11b (monocytes) and MAC3 (macrophages), but reduced the percentage of CD19 (B-cell marker) cell surface markers in α-phellandrene-treated groups, compared to untreated groups. α-Phellandrene promoted the phagocytosis of macrophages from blood samples at 5 and 25 mg/kg treatment and promoted natural killer cell activity from splenocytes at 25 mg/kg. Furthermore, α-phellandrene increased B- cell proliferation at 25 mg/kg with or without stimulation but promoted cell proliferation only at 25 mg/kg treatment with stimulation. Based on these observations, 25 mg/kg with α-phellandrene seems to have promoted immune responses in this murine model.*

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It is well-known that daily consumption of fruits and vegetables can reduce risk of oxidative stress and its damage on cells (1-3) and reduce the development of atherosclerosis (4-6) and cancer (7-11), and cardiovascular-associated diseases (12). Numerous studies have demonstrated that increased immune responses can increase the defense against microbial infection in healthy individuals and patients with cancer, including leukemia patients (13-15). Thus, numerous experiments have focused on investigating compounds from natural products for their effects on immune responses.

α-Phellandrene, a monoterpene, found in natural food sources and used in the food and perfume industry (16), is the major component of *Schinus molle* L essential oil (>50%) (16). There are few reports regarding the biological activities of α-phellandrene. It has been shown that α-phellandrene is not active as an anti-microbial agent (16, 17). However, there are no reports to show the effects of α-phellandrene on immune responses of normal mice *in vivo*. Thus, in the present study, we investigated the effect of α-phellandrene on the immune response of normal BALB/c mice *in vivo*.

Materials and Methods

Materials and reagents. α-Phellandrene and DMSO were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine and penicillin-streptomycin and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Carlsbad, CA, USA). α-Phellandrene was dissolved in DMSO at 1% and kept at –20˚C in a tube covered with black paper, to protect from light.

Male BALB/c mice. Fifty male BALB/c mice (aged eight weeks), around 22-25 g in weight, were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan, ROC) and maintained under specified pathogen-

free conditions at the Animal Center of the China Medical University. All animals were monitored and followed the institutional guidelines (Affidavit of Approval of Animal Use Protocol) that have been approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

Treatment of animals. A total of fifty male BALB/c mice were used for the whole experiment and were randomly separated into five groups of 10 animals. Group-I mice were treated with normal diet only, group-II mice were treated with olive oil (vehicle) as positive control; groups III, IV and V were treated with α-phellandrene at 1, 5 and 25 mg/kg in olive oil respectively, α-phellandrene was administered by oral gavage to the treatment groups at the above doses daily for 27 days. At the end of treatment, all mice were weighed and then sacrificed by euthanasia with CO_2 (18).

Immunofluorescence staining for surface markers of cells from each animal. At the end of treatment, each animal from each group was individually weighed before the blood was sampled. After grinding, spleen samples were removed and splenocytes were isolated for natural killer (NK) cell activity determinations. For surface marker measurements of leukocytes, 1 ml blood from all experimental mice was collected and lysed with 1× Pharm Lyse™ lysing buffer (BD Bioscience, San Diego, CA, USA) following the protocol from BD Biosciences. Blood samples from each group were centrifuged at 1500 ×*g* for 15 min at 4˚C to isolate white blood cells and then isolated cells were stained by the PE-labeled anti-mouse CD3, PE-labeled anti-mouse CD19, FITC-labeled anti-mouse CD11b and MAC3 antibodies (BD Biosciences Pharmingen Inc., San Diego, CA, USA) for 30 min before being analyzed by flow cytometry for determining the percentage of cell markers, as previously described (18).

Assays for phagocytosis by macrophages from each animal. Macrophages were isolated from (PBMCs) and the peritoneum of each mouse. Cells were added to 50 μl of *Escherichia-coli*-FITC according to PHAGOTEST® kit manufacturer's instructions (ORPEGEN Pharma Gesellschaft für Biotechnologische, Heidelberg, Germany), as described previously (18) and were analyzed by flow cytometry (BD Biosciences, FACSCalibration, Franklin Lakes, NJ) and were quantified by CellQuest software (18, 19).

Assays for NK cell cytotoxic activity. Approximately 1×10^5 splenocytes from each animal were placed in 1 ml of RPMI-1640 medium and then were cultured in a 96-well plate. YAC-1 cells (2.5×10^7 cells) (target of NK cells) in serum-free RPMI-1640 medium and PKH-67/Dil.C buffer Sigma-Aldrich Corp. (St. Louis, MO, USA). were added to the cells in each well and then mixed thoroughly for 2 min at 25˚C. PBS 2 ml was added to each well for 1 min followed by 4 ml medium and then incubated for 10–min before centrifuging at 1,500 rpm for 2 min at 25˚C. Each sample was measured for NK cell cytotoxic activity by flow cytometry as described elsewhere (18, 19).

Determination of T- and B- cell proliferation. Isolated splenocytes (1×10^5 cells/well) from each animal were placed in 96-well plates and then 100 μl of RPMI-1640 medium were added, and all were stimulated with concanavalin A (Con A, 5 μg/ml) for five days and lipopolysaccharide (LPS, 5 μg/ml) for three days for individual measuring T- and B-cell proliferation that were determined by using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), as previously described (18, 19).

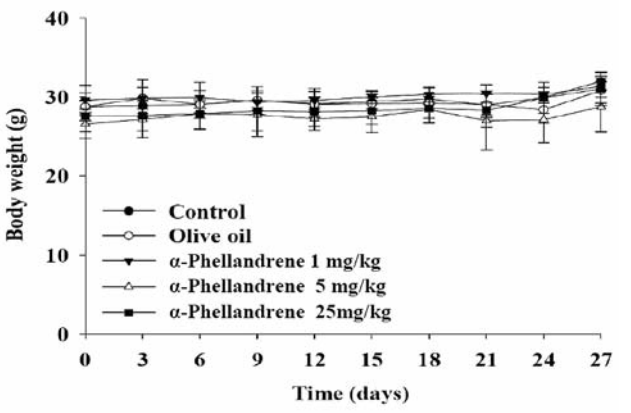

Figure 1. *Effect of α-Phellandrene on the body weight of normal BALB/c mice. Mice were divided into five groups; group I was treated with normal diet; group II was treated with olive oil; groups III-V were treated with 1, 5, 25mg/kg of α-phellandrene respectively. All animals were treated for 27 days. The total body weights were measured every three days.*

Statistical analysis. All data are expressed as the mean±S.D. of at least three experiments. Statistically significant differences between the positive control and α-phellandrene-treated groups were analyzed by Student's *t*-test. **p*<0.05 was used as the level of significance.

Results

α-Phellandrene affected the body weight of normal BALB/c mice. Treatment with α-Phellandrene did not significantly affect body weight (Figure 1) when compared to the mice given normal diet and the vehicle-treated mice.

α-Phellandrene affected cell markers of white blood cells. α-Phellandrene increased levels of CD3 (Figure 2A) at 1 mg/kg treatment, increased CD19 level (Figure 2C) at 5 and 25 mg/kg treatment, and increased Mac-3 level (Figure 2D) at 25 mg/kg treatment, but significantly suppressed the CD11b level (Figure 2B) at 5 and 25 mg/kg treatment when compared with the positive-control group. These results indicated that α-phellandrene significantly affected the cell population of normal mice *in vivo*.

α-Phellandrene affected phagocytosis by macrophages from PBMCs and peritoneal cavity of BALB/c mice. Figure 3 indicates that treatment with 5 and 25 mg/kg of α-phellandrene significantly promoted phagocytotic activity of macrophages obtained from PBMCs (Figure 3A) but not these obtained from peritoneal cavity (Figure 3B).

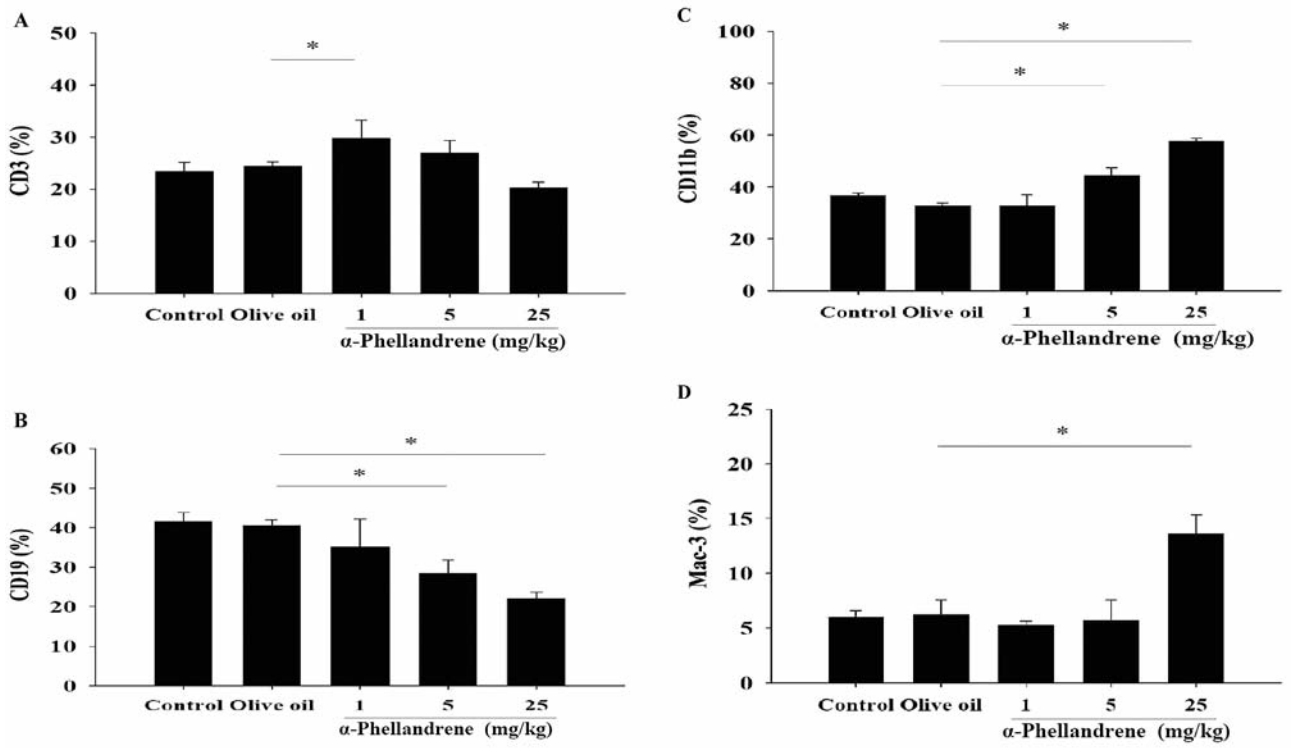

Figure 2. *α-Phellandrene affected the levels of cell markers in white blood cells from normal BALB/c mice. Blood was collected from each animal and was analyzed for cell markers (A: CD3; B: CD19; C: CD11b and D: Mac-3) by flow cytometry as described in Materials and Methods. The data are expressed as the mean±S.D. of three experiments (n=10). *p<0.05 Significant difference between control and α-phellandrene-treated groups.*

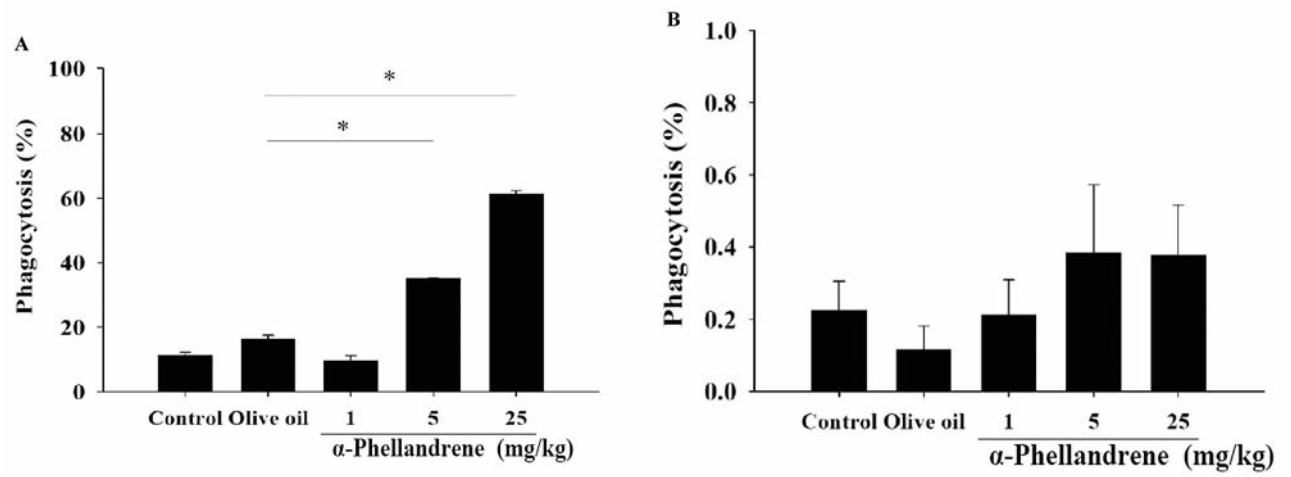

Figure 3. *α-Phellandrene promoted phagocytosis by macrophages from (PBMCs) and peritoneal cavity of normal BALB/c mice. Isolated macrophages were measured for phagocytosis by flow cytometery and quantified by CellQuest as described in the Materials and Methods. A: PBMCs; B: peritoneal cavity. *p<0.05 Significant difference between control and α-phellandrene-treated groups.*

α-Phellandrene affected the cytotoxic activity of NK cells and B- and T-cell proliferation in BALB/c mice. α-Phellandrene at 25 mg/kg promoted NK cell activity when compared to the control (Figure 4A) but did not at 1 and 5 mg/kg. The results for B- and T-cell proliferation are presented in Figure 4B and C, and indicate that α-phellandrene promoted B- cell T-cell proliferation both at 25 mg/kg. However, both low doses of α-phellandrene had no significant effects on cell proliferation.

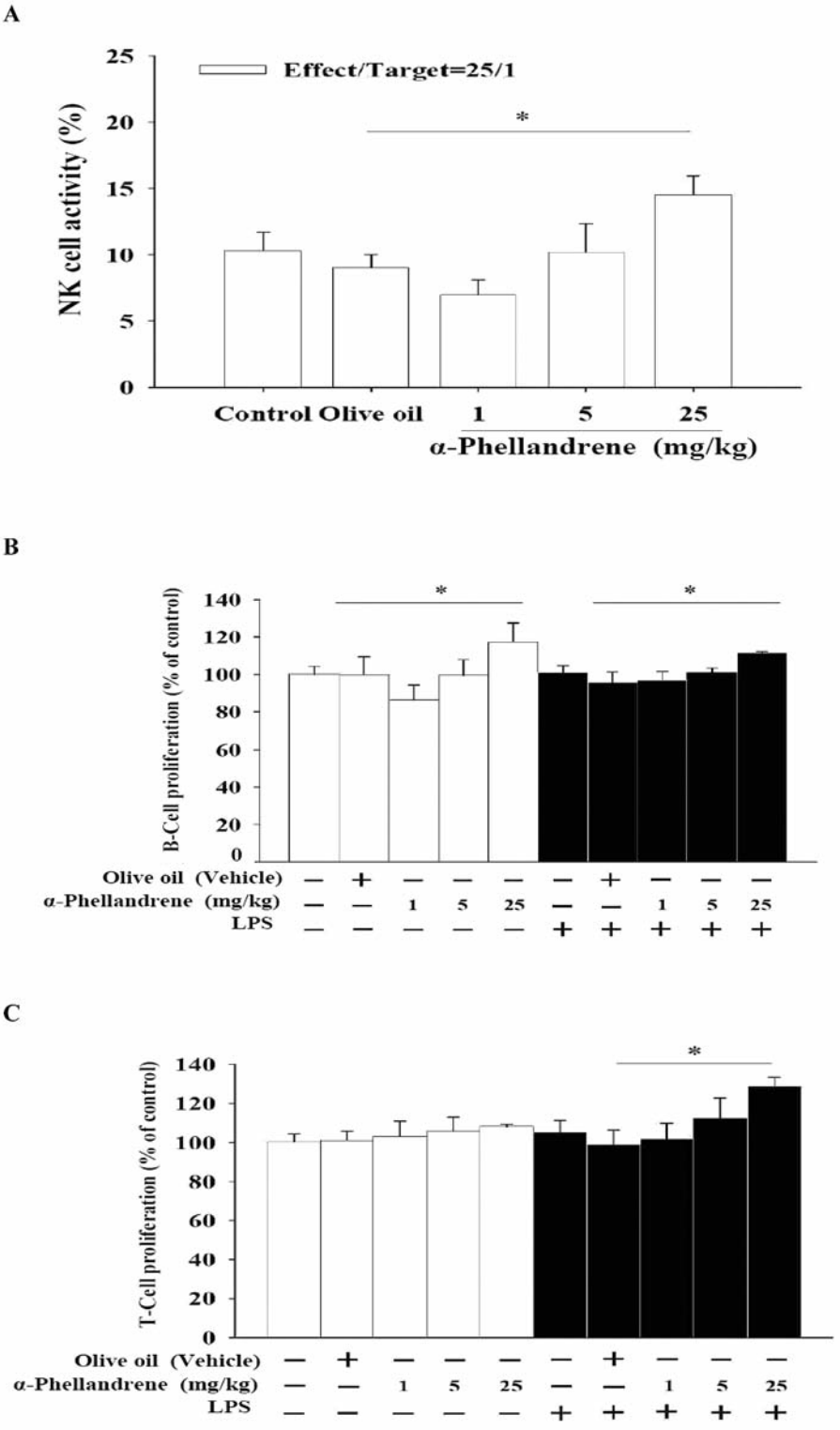

Figure 4. *α-Phellandrene affected the cytotoxic activity of natural killer (NK) cells and T- and B-cell proliferation of cells from normal BALB/c mice. Isolated splenocytes (1×10^5) were added to YAC-1 cells (2.5×10^7 cells) for the determination of NK cell cytotoxic activity by flow cytometry (A). B-Cells were pre-treated with (LPS) and then proliferation was analyzed by flow cytometry (B). T- Cells were pre-treated with (Con A) then cell proliferation was analyzed by flow cytometyr (C), as described in Materials and Methods. *$p<0.05$ Significant difference between control and α-phellandrene-treated groups.*

Discussion

Although only few reports describe the biological activities of α-phellandrene, several studies have shown that α-phellandrene did not present antimicrobial activity. There is no available information to show whether α-phellandrene affects immune responses in animals *in vivo*. Thus, herein, we investigated the effects of α-phellandrene on the immune responses of normal BALB/C mice *in vivo*. Treatment with α-phellandrene at 1, 5 and 25 mg/kg had no significant differences when compared to control groups. This may suggest that α-phellandrene did not induce toxic effects on normal animals. However, we found that α-phellandrene did affect cellular populations of immune-associated leukocytes, promoted macrophage phagocytosis, enhanced cytotoxic effects of NK cells and also promoted B- and T-cell proliferation.

Figure 2 demonstrates that α-phellandrene promoted and enhanced the cell population with CD3 marker (Figure 2A) at 1 mg/kg treatment. It is well-documented that T-cells play an important role in cell immune responses (20, 21) and without T-cells, there is no cellular or humoral immune responses in mice (20, 22). Furthermore, it was reported that HIV virus will first kill T-helper cells and then lead to acquired immune deficiency syndrome (AIDS) (23, 24). Figure 2C and D indicate that α-phellandrene increased the population of cells with CD11b at 5 and 25 mg/kg treatment and also that with Mac-3 marker. CD11b is a marker of monocytes and Mac-3 is a marker of macrophages, thus these results suggest that α-phellandrene promoted macrophage activity. It is well-documented that after antigen stimulation, macrophage phagocytosis promotes T-cell function and T-cells also released cytokines to help macrophage function (25, 26). In particular (Th1) cells play an essential role in the development of cell-mediated immunity to pathogens (27). Thus, α-phellandrene promoted macrophages (increased Mac-3 level), and we suggest that this occurs *via* T-cells (CD3) feedback to macrophages (Mac-3), leading to increased macrophage phagocytosis.

Our results also demonstrated that α-phellandrene reduced the population of cells with CD19 marker. CD19 is an activated B-cell surface marker (28, 29). Regarding the interaction of B-cells with other leukocytes from BALB/C mice after treatment with α-phellandrene, further investigations are needed. α-Phellandrene also significantly induced cell proliferation of T- and B-cells after Con A and LPS stimulation, respectively (Figure 4B and C).

It is well-known that activated macrophages play an important role in suppressing intracellular bacterial growth and in the resolution of infection (30). Our findings indicate that α-phellandrene promoted phagocytosis by macrophages from PMBCs in normal animals (Figure 3A), in agreement with results from the cell marker population with increased Mac-3 (Figure 2D). Based on these observation, we suggest that α-phellandrene stimulates macrophage proliferation (Mac-3) and promotes their function *in vivo*. Taken together, these results suggest that α-phellandrene promotes the immune response, in particular enhancing phagocytosis and NK cell activity through increasing the levels of T-cells, monocytes and macrophages in BALB/c mice *in vivo*.

Acknowledgements

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